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Isolation of Monocytes with High Purity and Yield from Peripheral Blood Mononuclear Cells by Flotation Density Gradient Centrifugation

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In this work, a simple, inexpensive and reproducible technique of flotation density gradient centrifugation was developed to isolate monocytes with high purity and yield from peripheral blood mononuclear cells (PBMC) using Histopaque solution, density and osmolarity of which were modified to 1.072 g/ml and 335 mOsm with phosphate-buffered saline (PBS) and sodium chloride (NaCl) solution, respectively. The average purity of monocytes was 74.75±3.84%, with the individual purity ranging from 71.44% to 82.38%. The average yield of monocytes was 32.62±11.16%, with the individual yields ranging from 21.02 to 53.63%. The monocytes isolated by floatation density gradient centrifugation could be successfully cultured into morphologically, phenotypically and functionally dendritic cells in vitro. In conclusion, the entire procedure seemed to be faster and more convenient, simple and cost-effective than other monocyte isolation methods, including plastic adherence and density gradient methods, and has the potential to be developed as a closed system for clinical scale generation of dendritic cells.

Key words : Flotation density gradient centrifugation, monocyte isolation

Introduction

Dendritic cells (DCs) play a crucial role in the induction of antigen-specific T-cell responses, and therefore have been studied for more than a decade as a vaccine in cancer patients with the goal of inducing a tumor-specific T-cell response. The most frequently described method for obtaining DCs is the ex vivo generation of DCs from $CD14^+$ monocytes. Although culture of plastic-adherent mononuclear cells remains one of the most popular method to isolate monocytes, monocytes can be obtained via density gradient centrifugation, magnetic bead selection of $CD14^+$ cells, depletion of B and T cells, or elutriation [14] Monocyte isolation by adherence is a laborious and time-consuming technique, which results not only in low yield (approximately less than 10% recovery), but also in the activation of the monocytes [17]. Other methods such as elutriation and fluorescence-activated cell sorting require the use of expensive instruments, which need technical skills [17]. The latter technique requires expensive antibodies and activates monocytes [17]. The use of magnetic beads conjugated to monoclonal antibodies is another expensive procedure for the isolation of monocytes. This approach can be used for both the positive [10,13] and negative selection [2,3] of monocytes. Negative selection of monocytes requires large quantities of beads [11], and in positive selection of monocytes, which is a more cost-effective approach, the monoclonal antibody (mAb)-bead complexes often cannot be removed from the isolated monocytes, thus interfering with the analysis of the cell surface markers to which the magnetic bead-conjugated mAb have bound and activating monocytes [9].

From the late 1960s through to the 1980s, many protocols for monocyte isolation were devised based on density gradient centrifugation, which is still an attractive and inexpensive method [7,12]. Although efficient separation proved difficult on the basis of density difference alone due to the overlapped densities of lymphocytes and monocytes, that monocyte purity can be improved by using a hyper-osmotic density gradient medium [5,8]. The principle is that as osmolarity increases, the cells expel water, thereby increasing their density and thus are able to pass a density barrier that otherwise they could not. Lymphocytes and monocytes can be separated because lymphocytes are slightly more sensitive to an increase in osmolarity than monocytes, and consequently lymphocytes tend to be smaller and more dense

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than monocytes [6]. It was shown that the higher the osmolarity, the higher the purity but the lower the yield of monocytes although the viability of the cells recovered at higher osmolarities was not affected [15]. A ready-made Nycodenz solution with density of 1.068 g/ml and osmolarity of 335 mOsm/kg is now commercially available. Therefore, for generation of DCs a density gradient centrifugation with Nycodenz solution was performed to isolate monocytes from PBMC or buffy coats. However, during isolation of monocytes with Nycodenz solution, it was difficult to obtain monocytes with high yields and purity.

In this study, an easy and simple method to isolate monocytes from PBMC was developed using Histopaque solution, density and osmolarity of which were modified to 1.072 g/ml and 335 mOsm with PBS and NaCl solution, respectively.

Materials and Methods

Blood collection

Peripheral blood was obtained from healthy volunteers following receipt of informed consent. For each donor, the blood was collected into a minimum volume of 350 ml of blood with triple bag containing citrate phosphate dextrose adenine-1 (CPDA-1).

Preparation of buffy coat

The blood pooled into a 400 ml triple bag was centrifuged at 550× *g* for 20 min at room temperature in a component R centrifuge with a swinging bucket rotor (Hanil Science, Korea). Buffy coat (30-40 ml) were collected and transferred into a fresh 50 ml polypropylene conical tube, and 500 µl of buffy coat were saved for analysis of complete blood count (CBC) and white blood cell (WBC) differentiation.

PBMC isolation

The buffy coat was diluted with same volume of PBS (Gibco-BRL Life Technologies Inc, Grand Island, NY). The 15 ml of histopaque-1077 was added to 50 ml conical tube, and the diluted buffy coat was loaded on the surface of histopaque-1077 carefully. The buffy coat-Histopaque was centrifuged at 400× *g* for 30 min at room temperature. No brake was applied during deceleration. Fractions of the resulting phases were collected as follows: first fraction: the first 18-20 ml of plasma, second fraction: the next 3 ml (approximately), including the PBMC band, third fraction: the next 10-12 ml (approximately), fourth fraction: cell pellet including red blood cell (RBC). The second fraction was collected and washed with 5 ml of PBS 3 times.

Monocyte isolation

To make an optimal density gradient solution, density and osmolarity of which were modified to 1.072 g/ml and 335 mOsm, respectively, 9.35 ml histopaque-1077 ((Sigma Aldrich, St Louis, MO) was mixed with 0.65 ml PBS and 50 µl 5 M NaCl solution. Collected cells were resuspended with 5 ml of the modified Histopaque solution thoroughly, transferred to 15 ml conical tube and centrifuged at 600× *g* for 20 min at room temperature. Four fractions including top band, upper middle fraction, lower middle fraction and bottom fraction were separately collected. Each collected fraction was washed with 10 ml of PBS 3 times (Fig. 1) .

Time that takes for monocyte isolation

1. Isolation of PBMC from a buffy coat on a histopaque-1077 density gradient: 60 min.
2. Cell counting: 10 min.
3. Preparation of a cell suspension with the desired cell concentration: 10 min.
4. Preparation of the modified Histopaque solution with 1.072 g/ml and 335 mOsm: 15 min
5. Separation of monocytes from PBMC on the modified Histopaque solution: 20 min.
6. Isolate the cells from 4 fractions and wash the cells: 15 min
7. Cell counting of each fraction: 10 min.

The procedure was summarized with a flow chart and

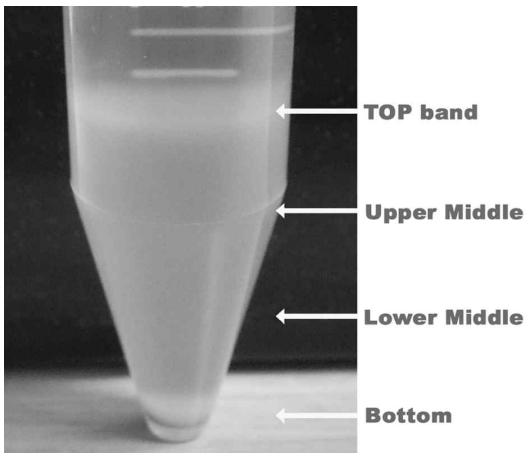

Fig. 1. A photograph taken after flotation density gradient centrifugation.

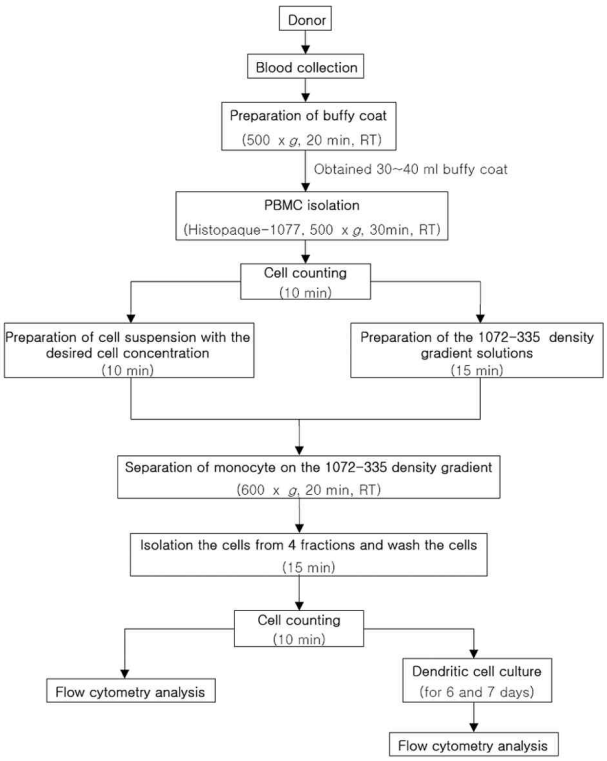

Fig. 2. Schematic flow chart of monocyte isolation procedure using the flotation density gradient centrifugation.

the overall time required was approximately 2.5 hr (Fig. 2).

Dendritic cell culture

DCs were effectively derived from monocytes that were enriched at Top band. The enriched monocytes were cultured at 1×10^6 cells/ml in serum-free CellGro medium (Freiburg, Germany) containing granulocyte macrophage colony-stimulating factor (GM-CSF; 1,000 U/ml, LG Life Sciences, Seoul, Korea) and interleukin-4 (IL-4; 1,000 U/ml, R&D Systems, Minneapolis, MN, USA). The cultures were performed in 75 cm^2 plastic flasks (Becton Dickinson, Franklin Lakes, N.J) at 37°C incubator with 5% CO_2. Fresh medium supplemented with cytokines was added every other day. On day 6 of culture, the cells were harvested to analyze surface markers and endocytosis of immature DCs. Immature DCs were matured for 1 day by treatment with tumor necrotic factor-alpha (TNF-α; 10 ng/ml; R&D Systems, Minneapolis, Minn.), interluekin-1beta (IL-1β; 10 ng/ml; R&D Systems, Minneapolis, Minn.), imterluekin-6 (IL-6; 1,000 ng/ml; R&D Systems, Minneapolis, Minn.) and prostaglandin E_2 (PGE_2; 1 μg/ml; Sigma). Matured DCs were harvested by vigorous pippetting with culture medium on day 7. Cell differentiation was monitored by light microscopy.

Preparation of cells for flow cytometric analysis

Flow cytometric analysis was performed to measure percentages and phenotypes of each mononuclear cells population.

1. Cells in the each fraction were phenotyped with the following fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or PE/cyanin (PC5)-conjugated mAbs: CD3, CD4, CD8, CD14, CD19 (Beckman Coulter).

2. DCs surface markers were examined at each maturation stage using the following FITC- or PE- conjugated mAbs : human leukocyte antigen (HLA)-ABC, HLA-DR, CD11c, CD14, CD54, CD80, CD83, CD86 (Beckman Coulter). Propidium iodide (PI) was used to determine viability with flow cytometry.

For each monoclonal antibodies combination, 100 μl cells were stained for 20 min at 4°C. Analysis was performed using the EPICS XL Flow Cytometer Coulter.

Endocytosis Assay with FITC-Dextran

DCs (1×10^6 cells/ml) were incubated at 4°C as a control and 37°C for 10 min. Dextran-FITC (40,000 MW, Sigma) was added at 1 mg/ml to each sample and incubated for a 60 min at the same temperature. Washing the cells twice in cold PBS quenched the endocytic activity and removed any free dextran-FITC. Cells were fixed in 200 μl of fixation buffer and analysed by flow cytometry.

Results

Assessment of monocyte purity and yield after flotation density gradient centrifugation

After flotation density gradient centrifugation with modified Histopaque (1072-335) cells appeared to be separated by density, showing at least three fractions consisting of a band between interface, a middle phase and a pellet (Fig. 1). After collection of four fractions including a top band (TOP), upper middle (UM) and lower middle (LM) fractions and a bottom pellet (BOT), the yield, recovery and purity of monocytes were determined with mAbs against CD3 and CD14, and summarized in Table 1, the values of which were obtained from 7 donors. Dot plots based on the flow cytometric analysis of donor 2, a representative experiment, were shown in Fig. 3. The mean percentage of monocytes ($CD3^-$ and $CD14^+$) in the starting PBMC suspensions was 14.28± 3.79% (mean±SD). Monocytes were highly recovered at TOP band. The average purity of monocytes

Table 1. The result of monocyte purity, yield and recovery of each fraction after the monocyte isolation procedure

Donor No.			1	2	3	4	5	6	7	Average±SD
Starting monocyte in PBMC suspension (% Monocyte)			11.89	12.10	10.83	16.57	17.70	20.14	10.76	14.28±3.79
Starting monocyte in PBMC suspension ($\times 10^6$)			61.83	34.00	51.98	57.73	69.03	82.57	88.62	63.68±18.55
Final monocyte enriched suspension	Monocyte Purity (% Monocyte)	TOP[a]	71.44	82.38	76.27	72.44	72.54	75.76	72.39	74.75±3.84
		UM[b]	12.94	8.84	11.27	21.56	18.71	24.45	21.53	17.04±5.99
		LM[c]	4.20	6.91	7.66	11.92	8.94	16.05	11.89	9.65±3.93
		BOT[d]	1.29	0.92	0.98	0.83	0.95	1.66	0.78	1.06±0.31
	Monocyte Yield ($\times 10^6$)	TOP[a]	15.22	18.23	16.78	22.96	14.51	20.76	28.23	19.53±4.87
		UM[b]	13.20	6.23	13.86	17.03	9.54	14.77	21.53	13.74±4.95
		LM[c]	4.28	6.05	13.33	14.42	11.73	16.05	9.63	10.78±4.37
		BOT[d]	1.44	0.40	1.26	0.85	1.50	1.66	1.13	1.18±0.43
	Monocyte Yield (%)	TOP[a]	24.62	53.63	32.28	39.78	21.02	25.14	31.86	32.62±11.16
		UM[b]	21.35	18.32	26.66	29.50	13.82	17.89	24.30	21.69±5.48
		LM[c]	6.92	17.79	25.64	24.98	16.99	19.44	10.87	17.52±6.86
		BOT[d]	2.33	1.18	2.42	1.47	2.17	2.01	1.28	1.84±0.52

The values were obtained from 7 donors. The average monocyte ratios of each fraction are presented the monocyte yield, purity and recovery. Cell suspensions of each fraction were analyzed by flow cytometry using forward scatter (FSC) vs. side scatter (SSC) dot plots gated on mononuclear cells.

[a]Top band after flotation density gradient centrifugation (monocyte enriched fraction)

[b]Upper Middle fraction after flotation density gradient centrifugation

[c]Lower Middle fraction after flotation density gradient centrifugation

[d]BOTTOM: Pellet after flotation density gradient centrifugation

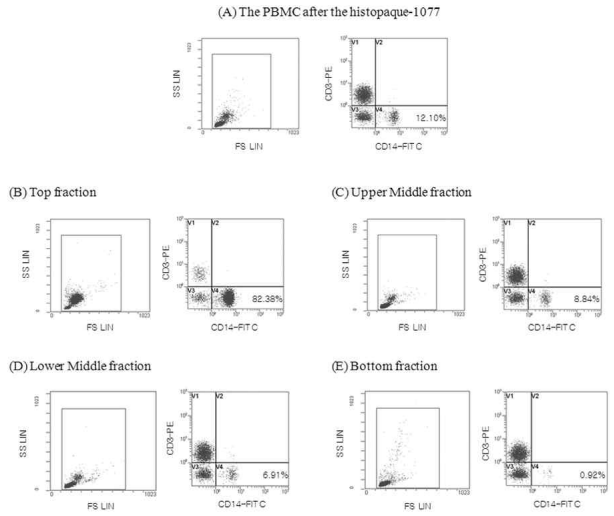

Fig. 3. The flow cytometric analysis of monocyte purity of each fraction after the flotation density gradient centrifugation. Forward scatter (FSC) vs. side scatter (SSC) and dot plots with CD3 and CD14 mAb from a representative donor #2 were shown. The PBMC after the histopaque-1077 step (A); the Top band (B), the Upper Middle fraction (C), the Lower Middle fraction (D) and the Bottom fraction (E) after flotation density gradient centrifugation step.

at the TOP band was 74.75±3.84%, with the individual purity ranging from 71.44% to 82.38%. The average yield of monocytes was 32.62±11.16%, with the individual yields ranging from 21.02 to 53.63%. The yield and purity were gradually decreased from top to bottom, showing lowest yield and purity in bottom fraction, as demonstrated in Table 1.

Phenotypes and endocytic activity of dendritic cells derived from monocytes isolated with flotation density gradient centrifugation

It was determined if DCs could be effectively generated from monocytes, which were enriched to TOP fraction after flotation density gradient centrifugation. The enriched monocytes at Top fraction seemed to be successfully differentiated into DCs. The cultured cells showed a typical immature DC morphology compared with monocytes (Fig. 4), and expressed typical immature DC markers including HLA-ABC, HLA-DR, CD11c, CD54 on and CD80, CD86 and CD83 on mature DCs (Fig. 5). After differentiation, CD14 monocyte marker was lost. The immature DCs derived from monocyte at Top fraction efficiently phagocytosed dextran (Fig. 6). These results demonstrated that the monocyte isolation method using flotation density gradient centrifugation did not affect the ifferentiation capacity and phagocytic activity of DCs.

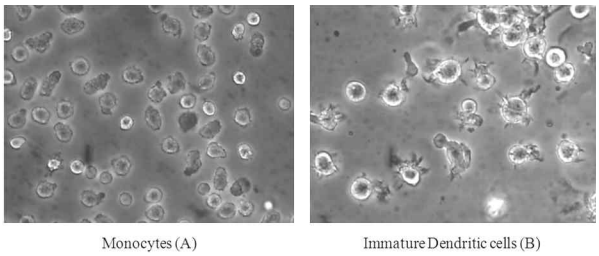

Fig. 4. Morphology of dendritic cells derived from enriched monocytes at Top band. (A) Morphology of the monocytes enriched at Top band after the flotation density gradient centrifugation. (B) Morphological features of immature DCs derived from monocytes enriched at Top band. The enriched monocytes were cultured for 6 days in Cellgro DC medium supplemented with IL-4 (1,000 U/ml) and GM-CSF (1,000 U/ml).

Discussion

For generation of monocyte-derived DCs enough for clinical study of anticancer vaccine, large amount of monocytes are required to be isolated in high yield and purity. Although culture of plastic-adherent mononuclear cells remains one of the most popular methods to isolate monocytes, monocyte isolation by adherence is a labor-intensive and time-consuming technique, involving incubation of PBMC for 1-2 hr in multiple tissue culture flasks, followed by manually washing off nonadherent cells. In addition, there are several other methods to isolate monocytes, such as magnetic bead selection of $CD14^+$ cells, depletion of B and T cells, or elutriation [14]. However, these techniques required the expensive instruments and technical skills.

In this work, a simple, inexpensive and reproducible technique of flotation density gradient centrifugation was developed to isolate monocytes with high purity and yield from peripheral blood mononuclear cells using Histopaque solution, density and osmolarity of which were modified to 1.072 g/ml and 335 mOsm with PBS and NaCl solution, respectively. This solution was constituted after several preliminary experiments. Monocytes were enriched at Top band fraction after flotation density gradient centrifugation in modified histopaque solution and the platelet contamination was almost negligible (not shown in data). The average purity and yield of monocytes at the TOP fraction were 74.75% and 32.62%, respectively. It is difficult to obtain monocytes with consistently high purity and yield using plastic adherence method due to variability of individual technical skills. Therefore, the flotation density gradient centrifugation method appeared to be less laborious and simpler and faster

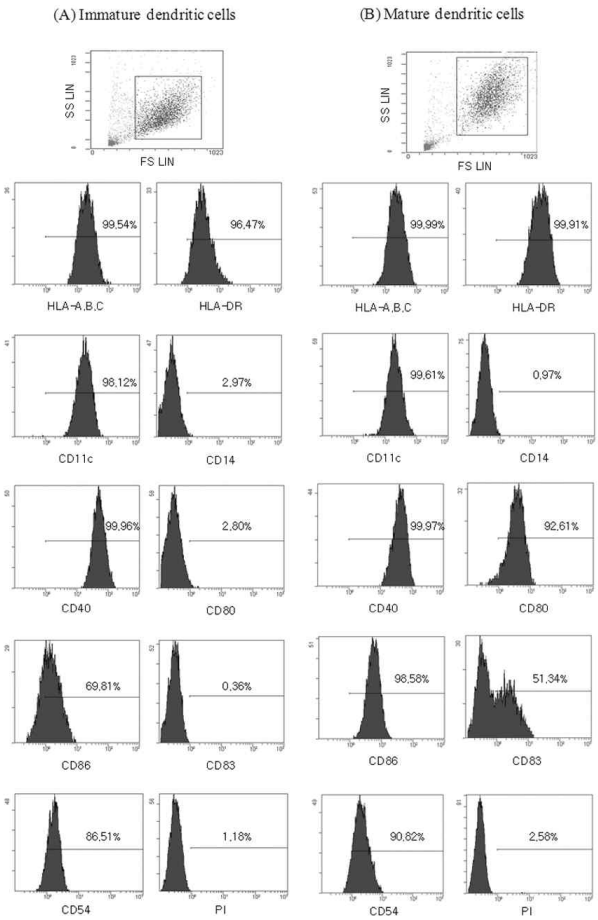

Fig. 5. Phenotypes of dendritic cells derived from enriched monocytes at Top band on the flotation density gradient centrifugation. Monocytes were cultured for 7 days in Cellgro DC medium supplemented with IL-4 (1,000 U/ml) and GM-CSF (1,000 U/ml). TNF-α (10 ng/ml; R&D Systems, Minneapolis, Minn.), IL-1β (10 ng/ml; R&D Systems, Minneapolis, Minn.), IL-6 (1,000 ng/ml; R&D Systems, Minneapolis, Minn.) and PGE_2 (1 μg/ml; Sigma) was added to induce maturation of dendritic cells on day 6. The histograms showed the expression of surface markers of the immature dendritic cells (A) and mature dendritic cells (B), as depicted in forward scatter (FSC) vs. side scatter (SSC) dot plots.

method than the plastic adherence method. Previously other laboratories used their unique density gradient centrifugation techniques. Centrifugation with OptiPrep density-gradient medium resulted in the average purity ranged from 87.9 to 96.4% and the mean yield, 26.1% [9]. However, this centrifugation gradient consisted of 3 layers, which need to prevent mixing of the layers. Recently, a simple and cost-effective Percoll density gradient had been developed to isolate monocytes from buffy coats [15]. The monocyte purity and recovery were about 75% and 13.5%, respectively.

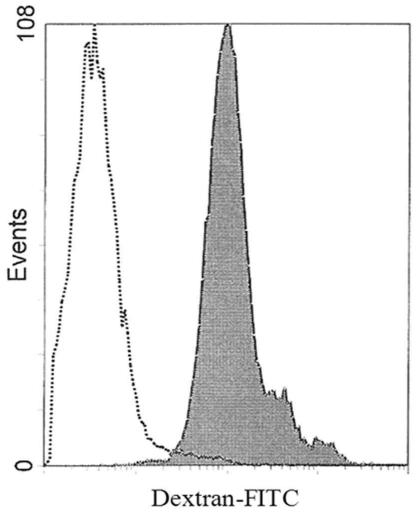

Fig. 6. Endocytic activity by immature dendritic cells. DCs (1×10^6 cells/ml) were incubated at 4°C as a control and 37°C for 10 min. Dextran-FITC (40,000 MW, Sigma) was added at a final concentration of 1 mg/ml to immature dendritic cells, and the cells were incubated for 1h at 37°C (thick lines) and 4°C (dotted lines) as a control. The values shown in the flow cytometry profiles were the mean fluorescence intensity indexes.

This procedure consisted of three steps: (1) the isolation of PBMC on a Ficoll density gradient; (2) the separation of monocytes from lymphocytes on a high density hyper-osmotic Percoll density gradient; and (3) the separation of monocytes from platelets and dead cells on a low density iso-osmotic Percoll density gradient. Basically, the flotation density gradient centrifugation method consisted of two steps including the isolation of PBMC on a Histopaque 1077 density gradient and the separation of monocytes from lymphocytes in the hyper-osmotic modified Histopaque solution, which can be made from commonly used solution including PBS and NaCl solution. This method showed comparable purity and yield with those of other previous density gradient techniques.

Other methods including immunomagnetic positive selection of CD14+ monocytes and elutriation method gave usually a very high purity (more than 80%) and recovery (more than 60%) of monocytes and are being developed as closed system for clinical-scale generation of DCs [1,4,16]. The monocytes isolated by floatation density gradient centrifugation could be successfully cultured into morphologically, phenotypically and functionally mature DCs in vitro. Since the monocytes can be easily transferred and cultured in bags, this method might have a possibility to be adopted as a quasi-closed system.

In conclusion, the entire procedure seemed to be more convenient, fast, simple and cost-effective than other monocyte isolation method including a plastic adherence and density gradient methods and would be needed to be developed as a closed system for clinical scale generation of DCs.

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초록 : 부유밀도구배 원심분리를 이용하여 말초혈액단핵구로부터 고순도 및 고수율의 단세포 분리방법

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본 연구에서는, PBMC로부터 고순도 및 고수율의 단세포를 분리하기 위해 Histopaque 용액의 density 및 osmolarity를 PBS와 NaCl 용액으로 1.072 g/ml 및 335 mOsm로 조절하여 간단하고 경제적인 방법인 부유밀도구배 원심분리법을 개발하였다. 위와 같이 조절된Histopaue용액을 사용하여 부유밀도구배 원심분리를 시행한 결과 단세포는 Top층에서 가장 많이 회수되었다. 회수된 단세포의 순도는 71.44~82.38%의 범위를 나타내었고 평균 74.75±3.84% 이었다. 또한, 수율은 21.02~53.63%의 범위를 나타내어 평균 32.62±11.16% 이었다. 이러한 방법의 부유밀도구배 원심분리에 의해 분리된 단세포를 수지상세포로 분화 유도시켜 그 형태, 표현형, 기능적인 면을 분석해 보았을 때 전형적인 수지상세포의 기능을 나타내었다. 결론적으로, PBMC로부터 단세포를 분리하기 위해 본 연구에서 사용된 부유밀도구배 원심분리 방법은 plastic adherence, elutriation 및 immunomagnetic selection보다 용이하고 경제적인 방법이다. 나아가 이러한 장점을 바탕으로 임상단계에서 수지상세포를 안전하고, 효율적으로 배양할 수 있는 closed system에도 적용이 가능할 것으로 기대된다.