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SCID-repopulating activity of human umbilical cord blood–derived hematopoietic stem and/or progenitor cells in a nonobese diabetic/Shi-SCID mice serial xenotransplantation model and immune cell activities in vitro: a comparative study of the filter method and the hydroxyethyl starch method

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BACKGROUND: A novel filter system was developed for umbilical cord blood (UCB) volume reduction. The aim of this study was to compare the functions of cryopreserved UCB cells processed by the filter and by the hydroxyethyl starch (HES) sedimentation method from the aspect of the graft quality.

STUDY DESIGN AND METHODS: UCB specimens were divided into two portions, processed in parallel by the filter or HES, and then cryopreserved in the clinical setting. The thawed UCB specimens containing 1×10^5 CD34+ cells were injected into nonobese diabetic/Shi-SCID mice, and the engraftment capacity in primary and secondary transplants was assessed. The functions of natural killer (NK) cells and monocyte-derived dendritic cells (DCs) were also assayed in vitro.

RESULTS: The percentage of recovery of CD34+ cells by both methods was equivalent. In the marrow of the primary transplant recipients, the percentage of hCD45+ cells in the filter group and HES group was 58.2 ± 31.6 and 46.5 ± 28.4 percent, respectively ($p = 0.016$). The engraftment capacity and multilineage differentiation in the secondary transplantations were equal in both groups. The cytotoxic activity of the NK cells and phagocytosis activity of the DCs from both the groups were similar.

CONCLUSION: The filter yielded a desirable percentage of recovery of hematopoietic cells with engraftment ability in the clinical setting. Thus, it is considered that the filter system may be useful for UCB banking for cord blood transplantation.

More than 3000 cases of unrelated cord blood transplantation have been performed to date, and the results suggest that human umbilical cord blood (UCB) is an excellent source of hematopoietic stem cells for transplantation in patients with various malignant and nonmalignant diseases.[1-4] Even now, there are more than a few patients who cannot receive cord blood transplantation owing to HLA mismatch. Expansion of the HLA library with extension of clinical cord blood banks would therefore be desirable.

Several centrifugation technologies for cord blood processing have been developed and have contributed to establishment of a foundation for clinical cord blood

ABBREVIATIONS: BM = bone marrow; CB-DC(s) = monocyte-derived dendritic cell(s) generated from fresh UCB specimen; DC(s) = dendritic cell(s); FTW = freeze-thaw-wash; NC(s) = nucleated cell(s); NK cell(s) = natural killer cell(s); TNC(s) = total nucleated cell(s); UCB = umbilical cord blood; VR = volume reduction.

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banking worldwide. In contrast, many kinds of filter devices, such as leukoreduction filters, have been developed and used for processing of blood in the field of blood transfusion medicine. We applied the filtration technology to UCB volume reduction (VR) and developed an innovative closed-circuit filter system[5,6] (StemQuick E, Asahi Kasei Medical Co. Ltd., Tokyo, Japan) that lends to simple handling and reproducible performance.

Meanwhile, a multilaboratory evaluation of procedures for reducing the volume of cord blood was performed by the BEST working party of the ISBT (International Society of Blood Transfusion). The study concluded that the filtration technologies yielded similar in vitro cell recoveries to the traditional centrifugation method.[7] The percentage of recovery of total nucleated cells (TNCs) and mononuclear cells (MNCs), however, were decreased by loading of the large number of TNCs. Therefore, we developed the novel filtration system with modification to provide a larger filtration area to improve the blood flow and to increase the retention capacity of TNCs.[8]

The objective of this study was to evaluate the functional utility of the filtration system. We compared several variables that affected the graft quality of cryopreserved and thawed UCB cells by use of matched paired UCB units processed in parallel by the filter method or the hydroxyethyl starch (HES) method developed by Rubinstein and colleagues.[9] Recently, to assess the self-renewal potential and multilineage differentiation ability of long-term-engrafting stem cells, many institutions have employed serial transplantation experiments.[10-14] In this study, with regard to compare the stem and/or progenitor cell functions between the two processing methods, we evaluated the engraftment capacity and/or multilineage differentiation ability in primary and secondary transplanted NOD/SCID mouse.

Until now, no comparative studies have been published between cell products processed by the filter and HES methods from the immunologic standpoint. We evaluated the recovery rates of monocytes, natural killer (NK) cells, B cells, and T cells after the VR, and compared the CD4-to-CD8 ratio in the CD3+ cell population between matched paired UCB units to investigate whether or not the filtration technology had some stimulatory effects on the T lymphocytes. Moreover, to evaluate the potential risk of reducing graft-versus-leukemia effect of NK cells by the filtration process, we compared the cytotoxic activity of the thawed MNCs derived with the filter method and HES methods in vitro. Dendritic cells (DCs) are professional antigen-presenting cells that have critical role for the induction of primary immune responses. This function affects autoimmune diseases, graft rejection, human immunodeficiency virus infection, and T-cell–dependent antibodies.[15] DCs play an important role in peripheral immunologic tolerance, which could have critical implications in the outcome of clinical transplantation as well.[16] Therefore, we generated monocyte-derived DCs from MNCs prepared from the cryopreserved UCB cells processed by the filter method (filter-DCs) or the HES method (HES-DCs) to compare functions of these DCs.

MATERIALS AND METHODS

Collection, processing, and thawing

Collection of UCB. The UCB donations were collected only from single-birth, full-term, low-risk deliveries with maternal informed consent, according to the international standards for UCB collection developed by NETCORD and the Foundation for the Accreditation of Hematopoietic Cell Therapy (FACT). The UCB units collected into a standard blood donor bag (Nipro Co., Osaka, Japan) containing 28 mL of citrate-phosphate-dextrose were stored at room temperature, transferred to the laboratory, and processed within 48 hours of collection.

Cell processing for the paired study in vitro and in vivo. The 11 units of UCB specimens with a net volume of more than 90 mL were divided into two portions, which were processed in parallel by the filter method or the HES method. The HES procedure, based on The New York Blood Center's protocol,[9] has been validated by the Tokyo Cord Blood Bank.

The novel filtration system, StemQuick R, and the recovery tool, SP-1 (both Asahi Kasei Medical), were used according to the manufacturer's instructions. To describe the principle of the procedure in brief, the nucleated cells (NCs) were trapped in the filter during whole-blood passage, and then the trapped cells were reverse-washed out into the recovery bag with a stroke of the syringe containing the recovery solution (23 mL of dextran 40 containing human serum albumin [final 2.8 wt/vol%]) and 15 mL of sterile air. The stroke of the syringe was controlled at constant speed and force by the SP-1. Three milliliters of 50 percent dimethyl sulfoxide (DMSO) in saline was added to each UCB unit processed with the filter and transferred to a 20:80 cryopreservation bag (Nipro).

For both the HES method and the filter method, the cryopreservation bags in the indicated canisters were applied in an automated freezer for UCB (BioArchive system, ThermoGenesis Co., Rancho Cordova, CA) to be frozen according to the programmed freezing procedure and cryopreserved in liquid nitrogen for at least 1 week before the date of transplantation.

Thawing and washing. On the day of transplantation, the paired samples processed with either the filter or the HES method were thawed quickly at 37°C. The UCB units were then transferred into a washing kit (Nipro) and diluted with HSA-dextran solution followed by centrifugation, in accordance with the two-step dilution protocol described by Rubinstein and colleagues.[9]

In vitro assays for the paired study

Cell counts. Cell counts were performed with an automated hemocytometer (XE-2100, Sysmex, Tokyo, Japan). The viability of the NCs was assayed by fluoromicroscopic analysis with ethidium bromide–acridine orange staining.[17]

Determination of the absolute CD34+ cell count. The absolute count of viable CD34+ cells was measured according to that described in the research application note "Absolute Stem Cell Counting Method (with Viability Option)." A TruCOUNT Tube (BD Biosciences, San Jose, CA) was used as an internal standard, and 7-aminoactinomycin-D was used to assess the viability of the cells.[18]

Clonogenic progenitor assay. Briefly, 1×10^5 NCs in a cord blood specimen and a murine bone marrow (BM) cell suspension were inoculated into medium (MethoCult 4434V or 4434, StemCell Technologies, Vancouver, British Columbia, Canada), respectively, and incubated for 14 days in plastic dishes in a fully humidified 5 percent CO_2 atmosphere. Colony-forming unit–total (CFU-Total) was scored by inverted microscopic examination.

NK cell functional assay. We used MNCs derived from the thawed UCB specimens by the above-described method for analysis of their cytotoxic activity against K562 (NK-sensitive) cells with a nonradioactive cytotoxic assay kit (CytoTox 96, LDH release methods, Promega, Madison, WI). Briefly, the effector cells and target cells were mixed to obtain effector:target ratios of 10:1, 5:1, 2.5:1, 1.25:1, and 0.625:1 for 4 hours in 100 μL of phenol red–free RPMI 1640 supplemented with 5 percent fetal bovine serum (FBS) in 96-well plates. As target cells were plated at 2×10^4 per well. Analysis and calculation were described elsewhere.[19]

In vivo assays for the paired study

NOD/SCID mice. Four- to six-week-old female NOD/SCID mice were obtained from CLEA Japan, Inc., and maintained under pathogen-free conditions in individually ventilated (high-efficiency particle-arresting filtered air) cages at the Experimental Animal Center, The Institute of Medical Science, The University of Tokyo (IMSUT), and provided with food and water ad libitum. The experimental protocol was given approval by the Animal Care and Use Committee of the IMSUT.

Paired NOD/SCID mice xenotransplantation. Paired NOD/SCID mice transplantation experiments were performed with 8-week-old female mice. For each of the seven cord blood experiments, paired HES-processed and filtered samples were thawed and washed in parallel, resuspended in RPMI 1640 containing 10 percent fetal bovine albumin at the cell concentration of 1×10^6 MNCs per mL, and transplanted into NOD/SCID mice. For each set of parallel transplantation experiments, two mice were injected with 1×10^5 viable CD34+ cells from the filtered sample and the HES-processed sample, respectively. Before the transplantation, the mice were irradiated sublethally with a dose of 240 cGy.

On every 11 days, 25 μL of rabbit anti-asialo GM1 antibody diluted in 500 μL phosphate-buffered saline (PBS) was injected into mice intraperitoneally. Then, 6 weeks after the transplantation, the mice were euthanized in a CO_2 chamber, and BM cells were obtained by flushing both femurs and tibias with 5 percent fetal calf serum in Iscove's modified Dulbecco's medium. The BM specimens were analyzed by the clonogenic progenitor assay, as described above and by flow cytometric analysis, as described below.

Engraftment analysis. Engraftment was analyzed by flow cytometry and the clonogenic progenitor assay. Samples of murine BM were incubated with anti-human CD45-phycoerythrin (PE) and costained with fluorescein isothiocyanate (FITC)-conjugated antibodies against human CD34-CD38, CD33, CD19, CD3, and CD41a (Becton Dickinson, Franklin Lakes, NJ). Isotypic controls were established with FITC- and PE-conjugated anti-mouse gamma globulin (anti-γ1–FITC and anti-γ1–PE, Becton Dickinson). NC subsets were determined by both their forward scatter and side scatter signals, followed by analysis of the cells for human CD45 expression together with the second FITC-labeled signal. Cells were considered positive if they were positive for both human CD45 and the corresponding second signal. The clonogenic progenitor assay was performed in accordance with a previously described procedure, with MethoCult 4434. Mice with less than 0.1 percent human CD45+ cells were considered to have nonengraftment.[20]

Second xenotransplantation. Serial transplantations were performed to assess the long-term engraftment capacity and self-renewal ability of the UCB cells processed by either the filter method or the HES method. BM specimens were harvested from both the hind femurs and the tibia of the two NOD/SCID mice that had already been primarily transplanted with the same UCB sample, analyzed, and mixed as a graft for secondary transplantation. For each set of parallel secondary transplantation experiments, two mice were injected with 1×10^7 NCs from the mixed BM specimen derived from the filter group, and two mice were transplanted with 1×10^7 NCs from that derived from the HES group. Before the transplantation, the mice were irradiated with a sublethal dose of 240 cGy. Six weeks after the transplantation, the mice were euthanized and BM was harvested from both hind femurs and tibias. Clonogenic progenitor assay and flow cytometric analysis were performed to evaluate the success of long-term engraftment. Means of the duplicated data were used for the statistical analysis.

In vitro generation of DCs and the functional assay

In vitro generation. Cord blood MNCs were obtained by density centrifugation with Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). A total of 3×10^7 cells of cord blood MNCs were seeded in 100-mm plastic culture plate (Primaria, Falcon, BD Labware, Franklin Lakes, NJ) in 4 mL of PBS and incubated for 2 hours at 37°C. Nonadherent cells were removed by extensive washing and adherent cells (approximately 90% nonspecific esterase staining) were subsequently cultured with granulocyte-macrophage–colony-stimulating factor (50 ng/mL) and interleukin-4 (IL-4) (50 ng/mL) in RPMI 1640 containing 10 percent heat-inactivated FBS for 7 days. To generate mature DCs, immature DCs were harvested and cultured with tumor necrosis factor-α (50 ng/mL) for an additional 4 days.

FACS analysis. For the expression profile of DCs, harvested immature or mature DCs were washed in PBS, and the cell pellet was incubated with the monoclonal antibodies (MoAbs) conjugated to FITC or PE for direct fluorescein: CD11c, CD14, CD40, CD80, CD83, CD86, HLA-ABC, and HLA-DR (all from PharMingen, San Diego, CA) for 30 minutes at 4°C. The stained cells were washed twice and suspended in PBS containing 0.2 μg per mL propidium iodide (Sigma Chemical Co., St. Louis, MO) to allow exclusion of dead cells before analysis by flow cytometer. After being washed with PBS, cells were analyzed with a flow cytometer (FACSCalibur, Becton Dickinson) and computer software (CELLQuest, Becton Dickinson).

Phagocytosis. Assay for phagocytosis of in vitro generated DCs have been described previously.[21] FITC-dextran (Molecular Probes, Eugene, OR) was added at a final concentration of 1 mg per mL into immature DCs, which were cultured for 60 minutes at 37°C. After incubation, these cells were washed four times with ice-cold PBS and analyzed by flow cytometry, as described above.

Statistical analysis

The values represent the mean ± standard deviation (SD). The nonparametric Wilcoxon's matched-pair rank test was used to compare the percentage of cell recoveries and engraftment capacities between the HES method and the filter method. For each group of two mice transplanted with the same sample, the mean percentage of human cells in the mice BM was calculated, and the products were used as data for the statistical analysis. A p value of less than 5 percent was considered to denote significance. The analyses were performed on a personal computer, with a statistical program software (JUSE-StatWorks/V3.0, The Institute of Japanese Union of Scientists and Engineers, Tokyo, Japan).

RESULTS

Eleven UCB units of large volume containing a total of 7.7×10^9 to 2.9×10^9 NCs were collected and processed within 12 to 42 hours from the time of collection. Because we divided each of the 11 UCB units into two portions, which were processed in parallel by the filter method and the HES method, we obtained 11 pairs of data sets before and after VR and after the freeze-thaw-wash (FTW). The recovery rates after the VR process and after the total (VR plus FTW) process were calculated. As for the recovery of TNCs and MNCs, there were significant differences between the two methods after both the VR process and the total process (Table 1). The VR process data were plotted into the X-Y graph, and the negative correlation between the number of TNCs loaded and percentage of recovery of TNCs was observed for the filter process (Fig. 1). The granulocyte content after VR of NCs was 59.3 ± 8.7 percent for the filter process and 63.3 ± 7.3 percent for the HES process ($p < 0.01$). The cell viability before VR was 94.5 ± 4.3 percent, and those after VR of NC by the filter method and by the HES method were 94.2 ± 3.8 and 92.5 ± 8.2 percent, respectively (NS, $p = 0.11$). The cell viability of NCs after the FTW process, however, was significantly higher when the filter method was used than when the HES method was used (79.0 ± 8.5% vs. 65.4 ± 9.0%, $p < 0.01$). The recovery rate of viable CD34+ cells and CFU-Total was equivalent for the

TABLE 1. Processing efficiency of the filter method and the HES method*

	VR			VR plus FTW		
	HES	Filter	P value†	HES	Filter	P value†
Recovery (%)						
TNCs	83.4 ± 4.0	66.6 ± 6.3	<0.01	66.5 ± 11.6	51.9 ± 5.5	<0.01
Granulocytes	82.6 ± 5.7	61.7 ± 8.2	<0.01	69.3 ± 5.1	50.7 ± 7.2	<0.01
MNCs	88.7 ± 4.1	78.0 ± 4.5	<0.01	72.1 ± 6.2	57.6 ± 5.2	<0.05
CD34+	89.8 ± 12.7	91.5 ± 20.7	NS	70.9 ± 7.7‡	65.4 ± 16.7‡	NS
Colony-forming cell	89.1 ± 14.0	84.6 ± 19.9	NS	44.2 ± 17.7‡	50.1 ± 17.2‡	NS
Reduction (%)						
RBCs	58.4 ± 11.0	88.3 ± 1.5	<0.01	75.8 ± 6.5	94.8 ± 0.7	<0.01
PLTs	55.9 ± 8.2	75.4 ± 4.9	<0.01	73.9 ± 1.6	90.8 ± 1.7	<0.01

* Data are shown as means ± SD (n = 11).
† Wilcoxon's matched-pair rank test was conducted for each pair.
‡ n = 9 owing to two samples not tested.

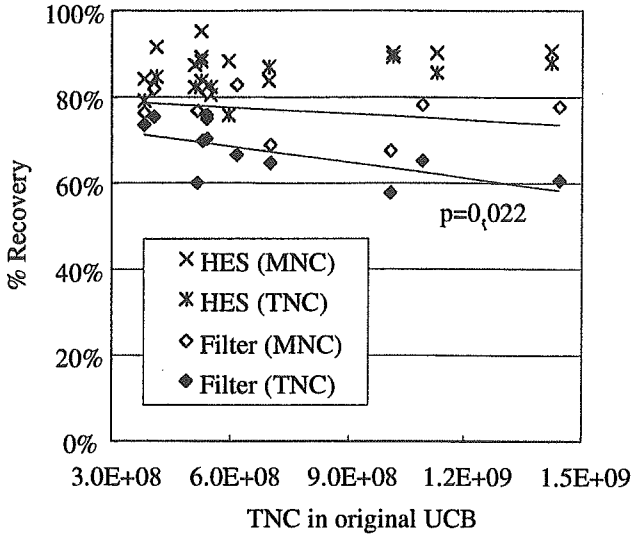

Fig. 1. Influence of the TNC number in the preprocessed UCB specimens on the percent recovery for of TNCs and MNCs. A negative correlation was observed for the samples processed by the filter method (TNCs).

TABLE 2. Recovery rates of WBC subsets during the VR process in the filter method and in the HES method*

Subset	Number	HES	Filter
CD3+	3	86.7 ± 13.8	74.4 ± 12.2
CD14+	3	110.0 ± 22.6	90.0 ± 9.0
CD19+	5	84.2 ± 15.6	77.8 ± 12.1
CD56+	5	83.7 ± 25.1	91.1 ± 22.3

* Data are shown as means ± SD.

filter and HES methods of processing (Table 1). The colony-forming cell ratio in original unprocessed UCB (before VR) was 0.11 ± 0.07 percent, and the ratios after the VR and after the FTW processes were 0.13 ± 0.08 and 0.08 ± 0.04 percent when the filter method was used and 0.11 ± 0.06 and 0.06 ± 0.05 percent when the HES method was used. The percentage recoveries of CD3+, CD14+, CD19+, and CD56+/CD3– cells after processing by the filter and HES methods were basically equivalent (Table 2). The CD4-to-CD8 ratio in the CD3+ cells of the unprocessed UCB and after VR by either the filter method or the HES method were almost the same (data not shown). Reduction of the counts of red blood cells (RBCs) and platelets (PLTs) after VR was significantly higher and more reproducible in the filter group than in the HES group. The total operation time for the VR process in the filter group was 19 ± 5 minutes; this was significantly shorter than that in the HES group (65 ± 8 min).

Cord blood hematopoietic stem cells processed by both the filter method and the HES method showed good engraftment results in primary and secondary NOD/SCID mouse transplants. The survival rates of both transplants

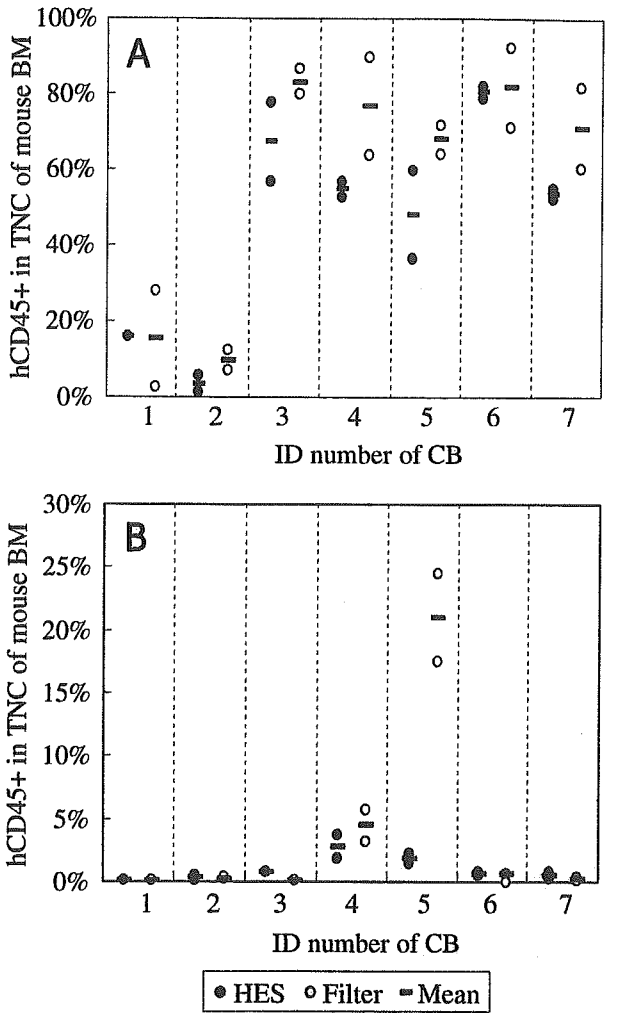

Fig. 2. Engraftment capacity in NOD/SCID mouse transplant recipients. (A) Primary transplant; (B) secondary transplant. (●) HES; (○) filter; (–) mean.

derived with the filter method and those derived with the HES method were 100 percent in both primary and secondary transplants (one mouse was omitted from the analysis because of death by suffocation after the cell infusion). The engraftment ratios of cells from the filter group and the HES group were 100 percent in primary transplants and 92.9 and 85.7 percent in secondary transplants, respectively. Mice with greater than 0.1 percent human CD45+ cells were considered to have engraftment.[20]

The engraftment capacities of the cells from the filter group and the HES group in primary transplants were 58.2 ± 31.6 and 46.5 ± 27.5 percent, respectively (n = 14 mice each). Each mean value for the filter group was slightly higher than that for the HES group (Fig. 2), and the differences were significant (p = 0.016). As for the secondary transplants, the engraftment capacities of the cells from the filter group and the HES group was 3.82 ± 7.76 and 1.02 ± 0.95 percent, respectively (n = 14 each). There

TABLE 3. Percentage of lineage markers in the total population of hCD45+ cells in the BM of primary and secondary NOD/SCID mouse transplant recipients*

	Primary transplant (%)			Secondary transplant (%)		
	HES	Filter	P value†	HES	Filter	P value†
CD3+	0.1 ± 0.2	0.3 ± 0.5	NS	0 ± 0	0 ± 0	NS
CD19+	43.4 ± 14.0	43.4 ± 17.7	NS	0.7 ± 0.7	2.3 ± 4.5	NS
CD33+	12.4 ± 10.2	14.1 ± 9.9	NS	0.8 ± 0.9	1.6 ± 2.2	NS
CD34+	9.7 ± 9.9	8.3 ± 6.4	NS	0.1 ± 0.1	0.4 ± 0.7	NS
CD34+/CD38–	2.8 ± 5.6	1.2 ± 1.8	NS	0.04 ± 0.04	0.03 ± 0.03	NS
CD41a+	1.5 ± 2.3	0.7 ± 0.6	NS	0.01 ± 0.03	0.02 ± 0.04	NS

* Data are shown as means ± SD (n = 7) of duplicate transplant data.
† Wilcoxon's matched-pair rank test was conducted for each pair.

was no significant difference between the mean values for the two groups.

From the results of the FACS analysis shown in Table 3, multilineage differentiation was observed for the cells obtained by either method in both primary and secondary transplants. In primary NOD/SCID transplants, the ratio of CD19+, CD33+, CD34+, and CD41a+ cells in hCD45+ cells in the harvested mouse BM was almost the same for both the filter and the HES group. Although some CD34+/CD38– cells were detected, their cell number was very low. In the secondary transplants, the similar results were obtained (Table 3). An example of the results of flow cytometric analysis is shown in Fig. 3.

The total number of BFU-E, CFU-GM, and CFU-Mixed per 1×10^5 BM TNCs for the filter group and the HES group in the primary transplants were 44.5 ± 8.3 and 40.6 ± 17.0 (NS) and in the secondary transplants were 4.1 ± 8.6 and 0.8 ± 1.0 ($p < 0.05$), respectively (Table 4).

The cytotoxic activity of filter-processed UCB-derived MNCs against K562 (NK cell–sensitive) cells was compared with that of the HES-processed UCB-derived MNCs. No cytotoxic activity was observed in the absence of incubation with IL-2 in either group for any effector:target ratio examined. With IL-2 incubation, however, the cytotoxic activity of the filter-processed UCB-derived MNCs was 102.7 ± 2.7 percent, which was almost the same as that of the HES-processed UCB-derived MNCs (98.6 ± 3.3%), with no significant difference at observed until an effector:target ratio of 10:1. In both groups, the results depended on the effector:target ratio (Fig. 4).

Immature and mature DCs were identified and gated as large cells based on forward-scatter and side-scatter analysis. Monocyte-derived DCs generated from fresh UCB specimens (CB-DCs) were used as control. Results showed that no difference was observed in the expression level of each antigen-presenting marker; HLA-ABC and -DR, costimulatory molecule marker; CD40, 80, 83, and 86, and DC1 marker; and CD11c and 14 among three DCs (Fig. 5). To assess the capacity of phagocytosis, DCs were exposed in FITC-dextran and its intensity was analyzed by flow cytometer. As shown in Fig. 6, although the filter-DCs or HES-DCs was slightly lower than that of CB-DCs, there were no significant differences among them.

DISCUSSION

As well as fast and simple operation under a closed-circuit environment, reproducible RBC and PLT reduction is one of the advantages of the filter system. Regarding the TNC and MNC recovery performance, the filter method showed statistically poorer results than the HES method. In contrast, the percentage recoveries of CD34+ cells and CFU-Total obtained by the filter method were comparable to those obtained by the HES method. The mean percentage of recovery of CD34+ cells was more than 90 percent by both methods, and this value is considered acceptable for the routine cord blood processing. Viability of the thawed UCB cells processed by the filter method was significantly higher than that by the HES method. There are two possible reasons for the phenomenon. One could be the lower content of granulocyte that is fragile for the freeze-thaw stress. Another could be the lower concentration of DMSO that is toxic for the cells.

Although the authors of the BEST working party in ISBT pointed out that processing by the filter method with StemQuick E was associated with a negative correlation between the number of TNCs loaded and the percentage recovery of TNCs and also MNCs, the results of this study with the modified filter did not show such a tendency in MNC recovery (Fig. 1). Our results may demonstrate that this filtration system is applicable for not only small number of TNCs loaded but also large number of them.

In our previous study, we performed paired transplantations of NOD/SCID mice of two divided UCB units that were processed either by the filter method or by the HES method.[6] A total 1×10^6 or 1×10^5 TNCs from thawed and washed units were transplanted. Although the interpretation of the data proved to be difficult because 22 of the 42 recipient mice died before the analysis, no significant difference in the hematopoietic reconstitution pattern was observed between the two groups of mice. To ensure statistical reliability, Eichler and colleagues[22] per-

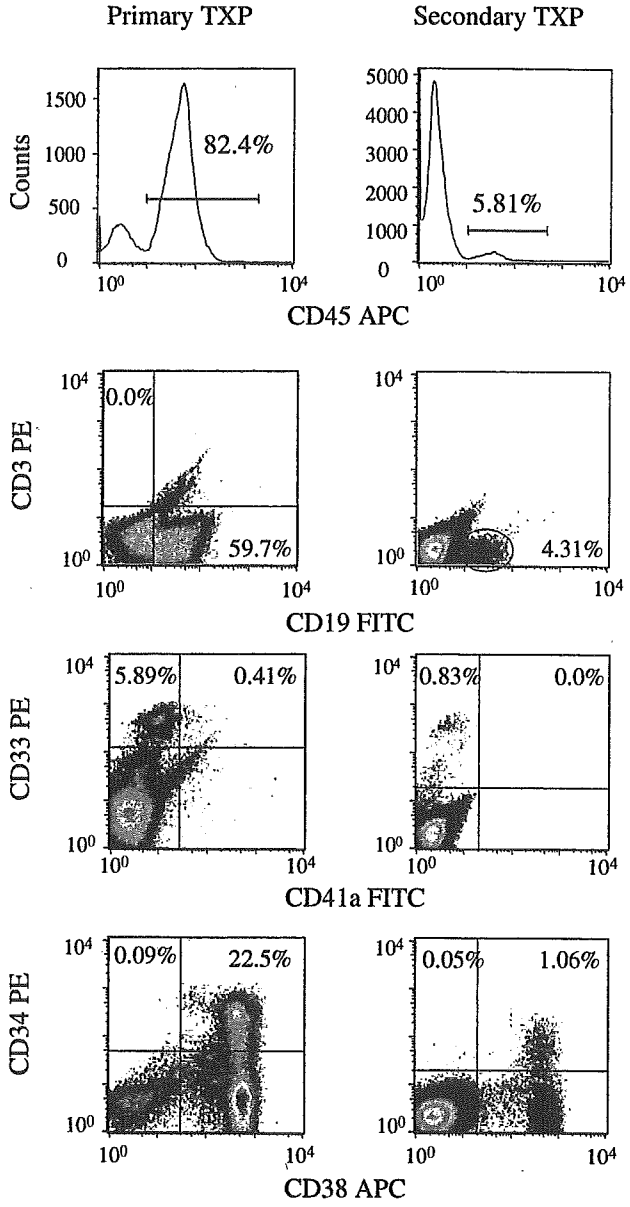

Fig. 3. Human cell engraftment in the BM of primary and secondary NOD/SCID mouse transplants transplant recipients and expression of lineage markers on human CD45+ cells in the BM of the secondary transplant recipient. UCB Sample 4 as a representative of seven different independent experiments is shown.

formed a detailed comparative study between the filter method and whole-blood cryopreservation method. They performed VR and cryopreservation with UCB units divided into two portions, each processed in parallel, either by the filter method or by the whole-blood method, and demonstrated that cord blood cells processed by the filter method had the same engraftment potential as that of the nonprocessed cells, with the NOD/SCID mice xenotransplantation model. Because the engraftment analysis was performed at 49 days after transplantation, however, this system was not valid to evaluate very primitive SCID-repopulating cells with self-renewal potential. In this study, we examined the engraftment efficiency and the multilineage differentiation activity in secondary NOD/SCID mice after serial transplantation. In primary and secondary NOD/SCID transplants, cells from the filter group showed equivalent or

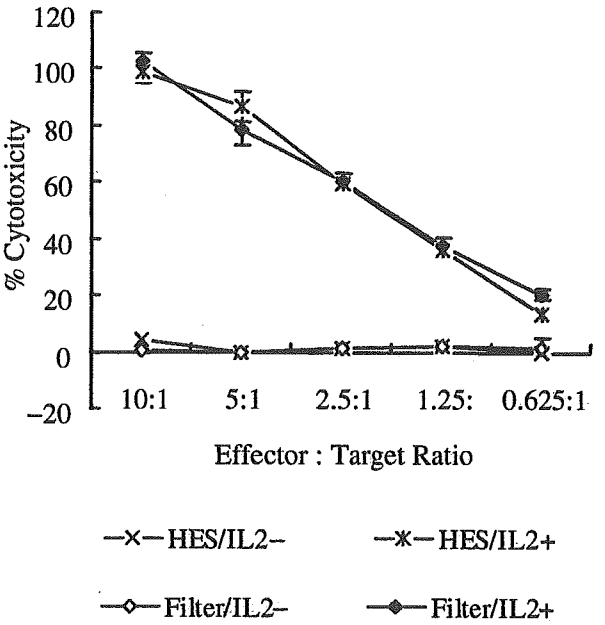

Fig. 4. Comparison of the cytotoxic activity against K562 cells of the MNCs derived from filter-processed UCB versus HES-processed UCB specimens (p = NS). Plus and minus symbols in the legend represent with and without incubation with IL-2 before the cytotoxic assay, respectively. Representative results from four independent experiments are shown.

TABLE 4. Number of human hematopoietic cell colonies per 1 × 10^5 NCs in the engrafted murine BM*

	Primary transplant			Secondary transplant		
	HES	Filter	P value†	HES	Filter	P value†
BFU-E	5.4 ± 2.6	5.9 ± 3.4	NS	0.1 ± 0.2	1.4 ± 3.3	NS
CFU-GM	30.8 ± 11.9	34.5 ± 4.8	NS	0.7 ± 0.8	1.9 ± 3.2	<0.05
CFU-Mixed	4.4 ± 3.7	4.0 ± 2.1	NS	0.03 ± 0.06	0.8 ± 2.0	<0.05
CFU-Total	40.6 ± 17.0	44.5 ± 8.3	NS	0.8 ± 1.0	4.1 ± 8.6	<0.05

* Data are shown as means ± SD (n = 7) of duplicate transplant data.
† Wilcoxon's matched-pair rank test was conducted for each pair.

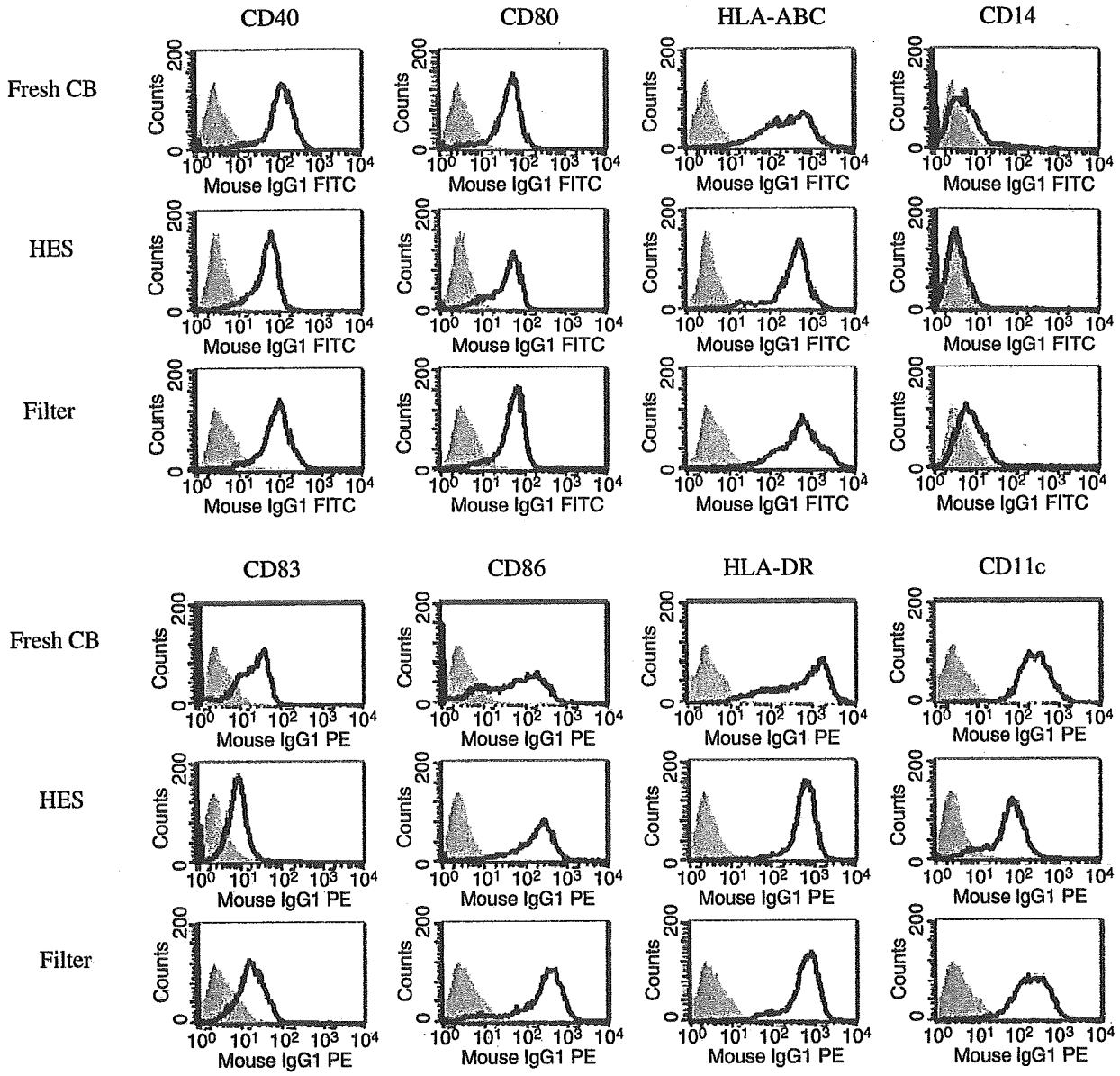

Fig. 5. Cell surface expression. Expression level of surface markers on filter- or HES-monocyte–derived DCs were stained with appropriately FITC- or PE-conjugated MoAbs and analyzed by a flow cytometer. These results are representative of three performed experiments with similar results. Cytogram colored in gray represents immature or mature DCs stained with anti-IgG1 MoAb conjugated with FITC or PE as an isotype control.

better engraftment results than those from the HES group, even for the same dose of CD34+ cells (Fig. 2 and Table 4). Lymphoid (CD19+) and myeloid (CD33+) differentiation was observed for both groups, and the ratio in BM TNCs was also almost the same (Table 3). Moreover, NCs in BM harvested from secondary NOD/SCID transplants were able to generate various types of human hematopoietic colonies in methylcellulose (Table 4). These results suggest that the filtration system can separate very primitive SCID-repopulating cells from UCB, and the separated cells show serial engraftment in NOD/SCID mice and differentiate into both myeloid and lymphoid progeny cells in the secondary transplant recipients.

NK cells have been identified as effector cells in the beneficial graft-versus-leukemia effect. NK cells derived from UCB have less cytotoxic activity than those derived from adult peripheral blood. When MNCs derived from fresh UCB were incubated with IL-2, however, the cytotoxic activity of the NK cells was enhanced and boosted to the level observed in NK cells derived from peripheral blood.[23] Recently, Nagamura-Inoue and associates[19] demonstrated that not only IL-2, but also IL-15, can induce cytotoxic activity in NK cells derived from thawed UCB. In

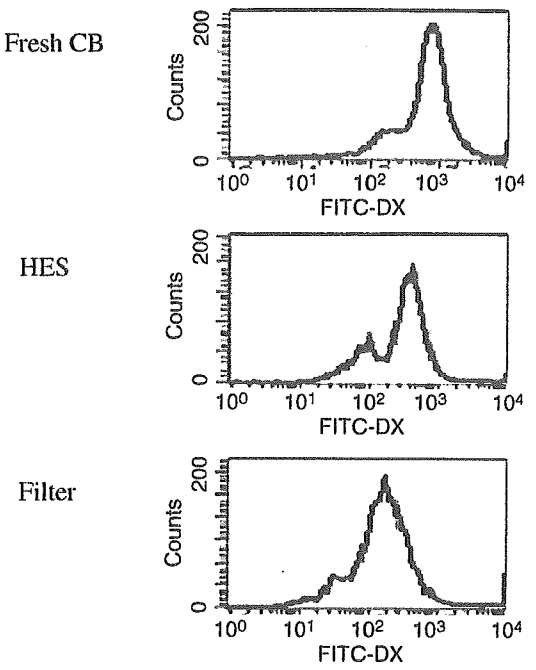

Fig. 6. Phagocytosis of immature DCs. Immature DCs were incubated with or without FITC-dextran for 1 hour. The capacity of phagocytosis was analyzed by flow cytometry. This result is representative of three experiments with similar results. Gray area represents cytogram of immature DCs without FITC-dextran as a control.

this study, we observed the same tendency as that reported in these studies with MNCs derived from both filter-processed UCB and HES-processed UCB. The cytotoxic activities of the two groups of MNCs were almost the same (Fig. 4). It is therefore reasonable to conclude that the filter process did not affect the cytotoxic activity of the NK cells in thawed UCB.

DCs are essential for initiating T-cell response against either host- or leukemia-specific antigens, suggesting that DCs derived from graft could be closely related to graft-versus-host disease and graft-versus-leukemia effect in cord blood transplantation. In this study, the expression of antigen profiles on CB-DCs, HES-DCs, or filter-DCs had almost similar levels at immature and mature stage (Fig. 5). There was no difference in the phagocytosis activity among all three groups (Fig. 6). A recent study and our previous data have demonstrated that phenotype and function of CB-DCs were almost similar to those of functional peripheral blood monocyte-derived DCs (data not shown).[24] Therefore, these results not only suggested that filter-DCs are also functional, but also implied that filter method did not influence differentiation of monocyte into DCs.

In conclusion, UCB was successfully volume-reduced by the filter method. Cryopreservation with a 25-mL cryobag could be appropriately performed in the clinical setting. UCB cells processed by the filter method had almost the same content of T cells, B cells, and NK cells as those processed by the HES method. The recovery rates of CD34+ cells and CFU-Total after the use of the filter method were equivalent to those following the use of the HES method. Therefore, the functional utility of the filtration system was demonstrated.

From the aspect of the functions of the cell product, UCB cells containing the same number of viable CD34+ cells processed by the filter method showed almost the same engraftment capacity and multilineage differentiation ability in primary and secondary NOD/SCID mouse transplants as those processed by the conventional HES method. Moreover, there were no disadvantages of the filter method compared to the HES method. As for NK cell activity and DC function, there was no difference in the activity of these cells between the two separation methods. Therefore, we concluded that the filtration system StemQuick R could serve as a safe and effective device for cord blood processing and that the stem cell product could be available for therapeutic use.

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