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HUMAN UMBILICAL CORD BLOOD: IMMUNOPHENOTYPIC HETEROGENEITY OF CD34+ HEMATOPOIETIC PROGENITOR CELLS

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ABSTRACT

Background. **Human umbilical cord blood (HUCB) is a possible alternative to bone marrow (BM) and mobilized peripheral blood (PB) for transplantation of hematopoietic progenitors. The aim of this study was to evaluate the phenotypic profile of CD34+ progenitors present in HUCB.**

Materials and Methods. **A flow cytometric analysis was performed on 20 HUCB samples, using a large panel of monoclonal antibodies recognizing different lineage or activation antigens, in double labeling with CD34.**

Results. **A total of 13,897±2,529 cells/μL, 0.84±0.83% of which were CD34+, was found. The large majority of CD34+ cells were committed toward initial myeloid differentiation (CD33+, CD13+) and expressed the transferrin receptor (CD71). A substantial proportion of these cells (about 40%) co-expressed CD45RA and CD117, while a very small number displayed markers of advanced myeloid commitment, such as CD14, CD15 and CD41 (less than 2%), or those of lymphoid differentiation: CD2, CD5, CD7, CD10 and CD19 (less than 6%). About 11% of HUCB CD34+ cells were *primitive* progenitors, as suggested by the absence of HLA-DR and CD38 on their surface.**

Conclusions. **As previously observed in BM and mobilized PB, the phenotype of HUCB CD34+ cells is quite heterogeneous. In particular, HUCB contains subpopulations of both early and committed hematopoietic progenitors which may represent a valid source for transplantation.**

Key words: human umbilical cord blood, hemopoietic progenitor cells, CD34, flow cytometry

The CD34 molecule is expressed on virtually all hematopoietic progenitors, including multipotent stem cells.[1-6] However, the number of detectable CD34-positive cells is very low in healthy donors; in fact, the CD34 antigen is expressed on 1-3% of bone marrow (BM) cells and on 0.1-0.4% of human umbilical cord blood (HUCB) cells, while only 0.01-0.1% of peripheral blood (PB) cells are CD34 positive. This low frequency can be greatly increased in PB samples from patients recovering from chemotherapy and/or growth factor administration.[7,8] These cells can be estimated by flow cytometry, collected on a large scale by cytapheresis, and cryopreserved for transplantation.[9,10] Recently, HUCB, which was shown to contain enough early and committed hematopoietic progenitors for hematopoietic reconstitution,[11,12] has been used for transplantation in children with various hematological disorders.[13,14] We and others have demonstrated that BM and PB CD34+ cell populations are extremely heterogeneous in surface antigenic expression.[15-19] The present flow cytometric study was designed to assess the phenotypic profile of HUCB CD34+ subpopulations.

Materials and Methods

Twenty unfractionated heparinized HUCB samples obtained from the umbilical vein immediately after vaginal delivery in uncomplicated term pregnancies were analyzed within 8

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Table 1. Monoclonal antibodies (MoAbs) used in this study.

MoAbs	*Cellular specificity*	*Source*
CD34 (HPCA-2)	Hemopoietic progenitors	BD
CD45 (Anti-HLe-1)	Leukocyte common antigen	BD
CD45RA (Leu-18)	Myeloid precursors	BD
CD45RO (Leu45RO)	Early progenitors, erythroid precursors	IT
HLA-DR (OK-DR)	Activated progenitors	OD
CD38 (Leu-17)	Activated progenitors	BD
CD71 (Transferrin receptor)	Activated progenitors	BD
CD117 (c-Kit receptor)	Stem cell factor receptor	IT
CD2 (Leu-5b)	T-lymphoid precursors	BD
CD5 (Leu-1)	T-lymphoid precursors	BD
CD7 (Leu-9)	T-lymphoid precursors	BD
CD10 (OK-BCalla)	B-lymphoid precursors	OD
CD19 (Leu-12)	B-lymphoid precursors	BD
CD13 (Leu-M7)	Myeloid precursors	BD
CD33 (Leu-M9)	Myeloid precursors	BD
CD14 (Leu-M3)	Myeloid precursors	BD
CD15 (Leu-M1)	Myeloid precursors	BD
CD41 (GpIIb/IIIa)	Platelet precursors	IT

BD: Becton Dickinson, OD: Ortho Diagnostic, IT: Immunotech.

hours of their collection. HUCB full blood counts were determined using a Coulter STKS (Coulter Diagnostics). Next, the samples were stained by the May-Grünwald-Giemsa method and an optical white cell differential count was performed. Leukocyte count was corrected for nucleated red cell contamination (a variable number of erythroblasts may be present in HUCB). The monoclonal antibodies (MoAbs) used in double labeling with fluorescein isothiocyanate (FITC)-labeled or phycoerythrin (PE)-labeled MoAb 8G12, directed against CD34 (HPCA-2), are listed in Table 1. One hundred µL of whole HUCB were simultaneously stained with 10 µL of the appropriate FITC- or PE-labeled MoAbs and then incubated for 30 minutes at 4°C in the dark. After red blood cell lysing (Lysing Solution, Ortho Diagnostic) and two washings by centrifugation in phosphate-buffered saline containing 0.1% sodium azide and 0.5% bovine serum albumin, the samples were analyzed by flow cytometry. Data were acquired on a FACSort flow cytometer (Becton Dickinson) equipped with a 15 mW argon laser emitting at 488 nm and Lysis II software. All channels were set for acquisition in the logarithmic mode.

CD34+ cell frequency estimation was performed on a total of 50,000 cells, using a combination of anti-CD45-FITC and anti-CD34-PE.[20] Two analysis gates were used. The first was set on a bivariate scattergram generated by combining side scatter (SSC) and CD45-FITC fluorescence (Figure 1a), drawn to include all CD45+ cells, thus excluding CD45-negative nucleated red cells. Thereafter this gate was used to generate a plot of anti-CD45-FITC vs anti-CD34-PE (Figure 1b). A second gate was drawn in the plot to include only CD34+ cells that formed a discrete cluster, as also shown in Figure 1b.

As the second step, an acquisition gate was set according to side light scattering cell properties and fluorescence intensity in order to collect only CD34+ cells, as previously described,[19] and 2,000 events were stored in list mode data files for two-color fluorescence (CD34+ vs myeloid, lymphoid or activation antigens). Mouse IgG1 and IgG2a (Becton Dickinson) were used as isotypic controls to determine background fluorescence. Only events that fell within the CD34 gate in forward versus side light scatter dot plots were accepted as CD34+ cells.

Results

The mean absolute number of total cells in HUCB was 13,897±2,529 µL (range 9,900-20,700 µL), while the mean absolute and proportional values of CD34+ cells were 116±121 µL (range 38-564) and 0.84±0.83% (range 0.3-4), respectively. Figure 2 shows the percentage of CD34+ subpopulations in the HUCB analyzed, expressed as mean±standard deviation.

With respect to myeloid commitment (Figure 2a), the coexpression of early myeloid antigens (CD13 and CD33) was detected on virtually all CD34+ HUCB cells. A significant subset of CD34+CD45RA+ cells (about 40%) was also found. Only a few progenitor cells displayed antigenic co-expression of other *mature* myeloid

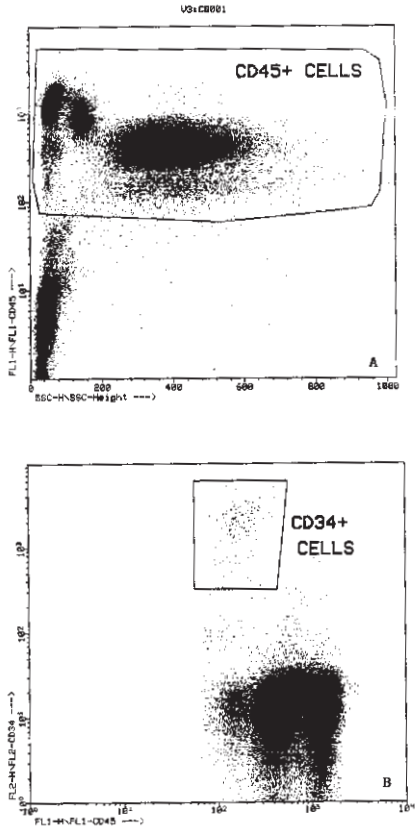

Figure 1.
1a: scattergram displaying CD45-FITC (y-axis) expression vs. side scatter cell properties (x-axis). An analysis gate was drawn to select CD45$^+$ cells only.
1b: scattergram displaying CD45-FITC expression (x-axis) vs. CD34-PE expression (y-axis). Only CD34$^+$ cells were gated.

surface molecules (CD14, CD15 and CD41). A very small subset of B and T lymphoid-committed CD34$^+$ cells (less than 6%) was found (Figure 2b). As depicted in Figure 2c, most of the HUCB CD34$^+$ cells coexpressed the transferrin receptor (CD71) and *c-kit* antigen (CD117), while CD45RO was found in less than 5% of CD34$^+$ cells.

The mean percentage of primitive hematopoietic progenitor cells (CD34$^+$CD38$^-$ and CD34$^+$ HLA-DR$^-$) was 10.6% and 12.6%, respectively (Figure 2c).

Figure 3 illustrates contour plots showing a typical coexpression pattern of CD33 and HLA-DR on CD34$^+$ cells in HUCB. The absolute levels of different CD34$^+$ subpopulations in HUCB are reported in Table 2.

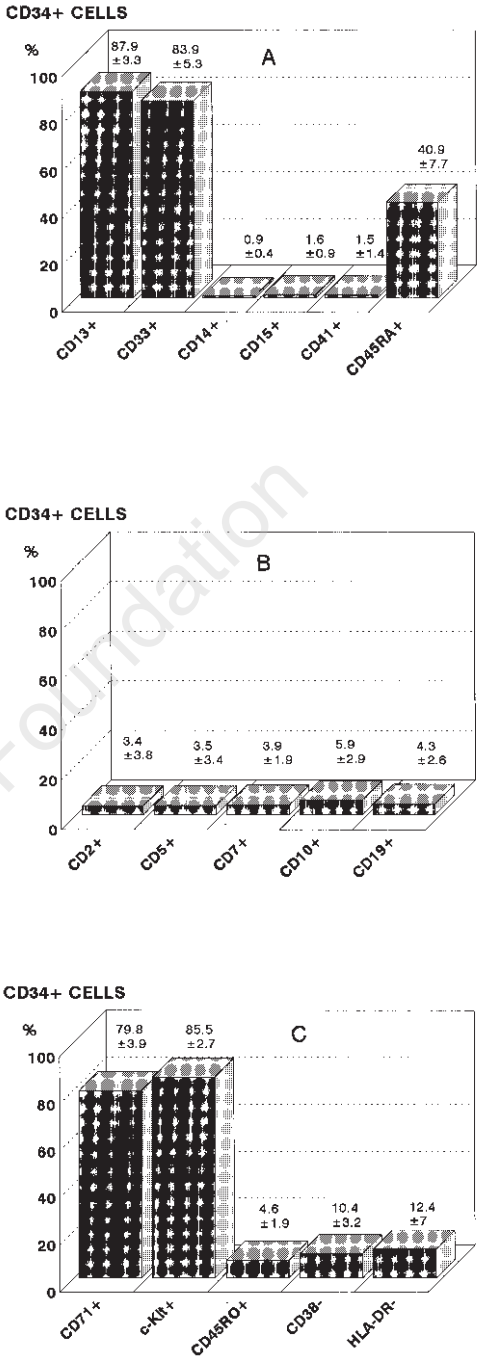

Figure 2.
A. Myeloid commitment. Bar graphs show mean (± standard deviation) proportional values of myeloid antigen co-expression on CD34$^+$ cells in HUCB.
B. Lymphoid commitment. Bar graphs show mean (± standard deviation) proportional values of lymphoid antigen co-expression on CD34$^+$ cells in HUCB.
C. Other antigens. Bar graphs show mean (± standard deviation) proportional values of other antigen co-expression on CD34$^+$ cells in HUCB.

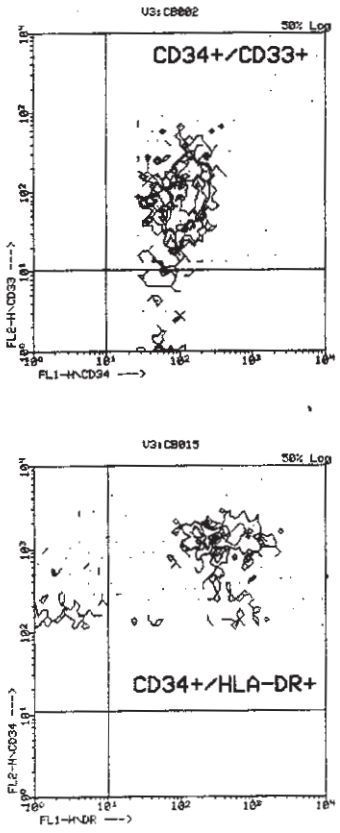

Figure 3. Representative contour plots showing examples of CD34 (-PE or -FITC) co-expression with CD33-PE (upper) and HLA-DR-FITC (lower) in HUCB.

Table 2. HUCB CD34+ progenitor cell subsets: absolute values.

CD34+ subsets	*Mean values/μL*
CD34+ total	116±121
CD34+CD38−	10.1±6.1
CD34+HLA-DR−	12±11.2
CD34+CD45RO+	6.6±6.8
CD34+CD45RA+	33.7±19
CD34+c-Kit+	111.3±140
CD34+CD71+	76.1±39.1
CD34+CD13+	98±116
CD34+CD33+	102±101
CD34+CD14+	0.6±0.5
CD34+CD15+	0.9±1.6
CD34+CD41+	0.8±0.8
CD34+CD2+	2.9±2.6
CD34+CD5+	3.2±5.8
CD34+CD7+	2.8±2.3
CD34+CD10+	4.9±4.5
CD34+CD19+	2.7±1.5

The values are expressed as mean (± standard deviation) number of CD34+ cell/μL of HUCB.

Discussion

Our study confirms that CD34+ cells detectable in fresh HUCB have a heterogeneous immunological profile. The large majority of CD34+ cells were found to be committed toward initial myeloid differentiation (CD13+ and CD33+). Only a few CD34+ cells co-expressed CD14, CD15 and CD41 antigens (late myeloid commitment), while a more considerable proportion of HUCB CD34+ cells showed surface expression of CD45RA and *c-kit* receptor (CD117), which are now also considered as myeloid markers.

A very small number of both T and B lymphoid-committed CD34+ progenitor cells was observed. On the contrary, the majority of CD34+ cells were found to be positive for the transferrin receptor (CD71), which is highly expressed on early erythroid progenitors and, albeit to a lesser extent, on proliferating cells.

CD34+ hematopoietic progenitor cells lacking CD38 and HLA-DR antigen expression are enriched for primitive stem cells, as demonstrated by previous reports. These cells are thought to be responsible for long-term engraftment of transplanted hematopoietic progenitor cells. Several studies[21-26] have suggested that the majority of long-term culture-initiating cells belong to the CD34+CD38− and CD34+HLA- DR− subsets. However, Traycoff and co-workers have recently shown that in HUCB these very early progenitor cells reside in the CD34+HLA-DR+ cell fraction instead.[27-29] In our hands about one tenth of the total CD34+ cell population was CD34+CD38− and CD34+HLA-DR−. In our experience, this proportion is greater than that observed in BM and mobilized PB CD34+ progenitor cells.[19] Thus, according to immunological features, HUCB seems to be an optimal source for transplantation because of its consistent percentage of both uncommitted and myeloid-committed cells that potentially provide the long-term and short-term engraftment, respectively, needed for a safe hematopoietic transplantation. However, in this context, the possible contribution to the

hematopoietic reconstitution of the recently described $CD34^{-}CD117^{+}$ stem cells should also be considered (Lanza F, personal communication).

To date, more than 150 patients have been transplanted with HUCB stem cells.[30] In these patients, the mean time to neutrophil and platelet recovery was longer than that observed after BM or mobilized PB rescue. Indeed the autologous $CD34^{+}$ cell threshold dose needed for safe engraftment is not well established. Some authors indicate a dose of 2×10^{6}/kg $CD34^{+}$ progenitor cells.[31,32] However, more recently it has been claimed that $CD34^{+}$ cell doses lower than 5×10^{6}/kg, although frequently successful, expose a fraction of patients to the risk of delayed or defective platelet reconstitution.[33] In this regard, doses higher than 8×10^{6}/kg are probably required for the best results in terms of rapid, complete and sustained hematopoietic reconstitution of myeloablated hosts.[34,35] There are also some controversies about the number of $CD34^{+}$ hematopoietic cells in HUCB required to engraft older children over 40 kg in weight and adult recipients.[36,37] The mean value of HUCB collected after normal full-term deliveries is about 100 mL.[14] According to our data (mean HUCB cellularity was 13,897±2,529 μL, of which 0.84±0.83% were $CD34^{+}$ cells), a 100 mL HUCB sample contains a mean of 0.3×10^{6}/kg $CD34^{+}$ cells for a 40 kg recipient; thus HUCB collected from a single placenta does not seem to contain sufficient progenitors to ensure a safe engraftment in adults. However, as suggested by clinical and experimental studies, engraftment may not depend only upon the number of stem progenitors, but also upon their quality. In this setting, the phenotypic characteristics of HUCB progenitors are of particular interest. In the future *ex vivo* expansion techniques of $CD34^{+}$ progenitor cells[38-40] will probably resolve these problems and will perhaps permit us to consider HUCB as the most important source of hematopoietic progenitor cells for allogeneic transplantation.

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