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Flowcytometric analysis of aldehyde dehydrogenase activity in mononuclear cells from umbilical cord blood

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Aldehyde dehydrogenase (ALDH) is a cytosolic enzyme that is responsible for the oxidation of intracellular aldehydes. Elevated levels of ALDH have been demonstrated in murine and human progenitor cells compared with other hematopoietic cells, and this is thought to be important in chemoresistance and purification techniques and an indication of the proper function of the cell. A Flowcytometric method for the assessment of ALDH activity in viable cells recently has been developed. Forty six cord blood samples from mothers which underwent normal delivery of full term infants were obtained, after informed consent. Mononuclear cells were obtained by Ficoll-Paque density centrifugation and ammonium chloride red cell lysis. Percentage of viable cells was determined by trypan blue exclusion dye. Cells were labeled with Aldefluor reagent (Vancouver Canada) as described by the manufacturer. Cells were then stained with phycoerythrin (PE)–conjugated anti-CD34 (Miltenyi Biotec, Cologne, Germany) antibodies for 30 min at 4°C. Cells were washed and re-suspended in phosphate-buffered saline (PBS) with 2% fetal calf serum. Cells were then analyzed on coulter epics flow cytometer. The mean percentage of ALDH enzyme expression among the CD34+ cells in the cord blood samples was 61.3% with a minimum of 28% and a maximum of 94.6%. Significant correlations were found between the white blood cell (WBCS) count in the cord blood samples and both the CD34+ cell count and the count of ALDH expressing cells, while no correlation was found between the CD34+ cells count or the ALDH expressing cells count in the cord blood samples and either the sex or the weight of the newborn. Identification and isolation of cells on the basis of ALDH activity provides a tool for their isolation and further analysis. In **summary, a high ALDH-1 activity identifies CD34$^+$ cells in cord blood.**

Key words: Umbilical cord blood, stem cells, aldehyde dehydrogenase (ALDH), CD34.

INTRODUCTION

Aldehyde dehydrogenase (ALDH) is a family of enzymes involved in metabolism of aldehydes to their corresponding carboxylic acids (Cheung et al., 2007). It plays an important role in metabolism of vitamin A as well as in mechanisms of resistance to alkylating agents, for example cyclophosphamide (Storms et al., 1999). For these reasons, ALDH is considered a protecting or detoxifying enzyme, able to preserve stem cells from cytotoxic effects (Storms et al., 1999; Fallon et al., 2003; Hess et al., 2004). One of the accepted technologies to

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identify human hematopoietic stem cells (HSC) is based upon flow cytometry (FCM) detection of ALDH enzymatic activity (Storms et al., 1999).

The functional role of ALDH has been studied, with specific inhibitor of ALDH, diethylaminobenzaldehyde (DEAB), which was able to alter the molecular and cellular mechanisms that control self-renewal capacity of human HSC (Chute et al., 2006). The evidence of ALDH involvement in the physiology of HSC was further highlighted by a series of studies devoted to purification/analysis of highly immature progenitor cells, particularly in human cord blood (CB) as well as in murine bone marrow (BM) (Christ et al., 2007; Juopperi et al., 2007; Hess et al., 2006). The importance of ALDH in human hematopoiesis was also testified by a recent study in which the authors tried to purify HSCs by combining FCM cell sorting and Hoechst-33342 efflux ability (the so called "side population") (Pearce and Bonnet, 2007). At variance with previous findings obtained in mouse, human BM hematopoietic cells able to exclude Hoechst-33342 did not correspond to highly immature HSCs. On the other hand, the authors proposed that ALDH activity had to be considered as the reference method for the detection of immature HSCs in human BM, at the same time emphasizing the need for studies about expression pattern of ALDH in comparison with other hematopoietic cell markers in this tissue (Pearce and Bonnet, 2007).

Human HSCs have traditionally been characterized by the expression of cell surface markers such as CD34 (Civin et al., 1984; Bhatia et al., 1997a), but not all human hematopoietic repopulating cells express CD34 (Bhatia et al., 1998; Dao et al., 2003) and cell surface phenotype can be altered by cell cycle progression and *ex vivo* manipulation (Dorrell et al., 2000; Guenechea et al., 2000; Bhatia et al., 1997b; Hess et al., 2003; Nakamura et al., 1999; Sato et al., 1999). A purification strategy complementary to the use of surface phenotype involves the assessment of intracellular enzyme activities associated with the protection of primitive cells from oxidative insult during hematopoietic development. One promising purification strategy exploits cytosolic ALDH, an enzyme implicated in retinoid metabolism and the resistance of HSCs to alkylating agents such as cyclophosphamide (Sahovic et al., 1988; Takebe et al., 2001). Murine repopulating cells (Sharkis et al., 1997; Jones et al., 1995) and human hematopoietic progenitors have previously been isolated based on increased activity of intracellular ALDH (Storms et al., 1999; Jones et al., 1995).

One promising strategy is HSC isolation according to a conserved stem cell function rather than phenotype. In the murine system, lymphohematopoietic stem cells have been isolated according to the high expression of the detoxifying enzyme (ALDH) (Sharkis et al., 1997; Jones et al., 1995; Jones et al., 1995). Storms et al. (1999) described a fluorescent substrate of ALDH (termed aldefluor) that can be used to isolate cells with increased ALDH activity by fluorescence-activated cell sorting (FACS). The substrate is an amino acetaldehyde molecule conjugated to a BODIPY (4, 4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza- 5-proprionic acid) fluorochrome that is metabolized by ALDH to an aminoacetate anion was retained within the cell because of its negative charge. Thus, the amount of fluorescent product that accumulates in viable cells correlates to ALDH activity and cells with high ALDH activity can be selected from human umbilical cord blood (UCB) or mobilized peripheral blood by FACS (Fallon et al., 2003; Storms et al., 1999). UCB cells isolated by using this strategy have demonstrated to be depleted of lineage committed hematopoietic cells and are enriched for primitive hematopoietic progenitors detected in clonogenic *in vitro* cultures (Storms et al., 1999). This approach has allowed the analysis of viable murine and human ALDH+ progenitors by flow cytometry (Storms et al., 1999).

MATERIALS AND METHODS

We obtained forty six cord blood samples from mothers attending Suez Canal University Hospital, after informed consent. All cord blood samples (each sample = 30 ml) were stored overnight at room temperature before ALDH analysis. The protocol was approved by the institutional research ethics committees. Mononuclear cells (MNCs) were obtained by Ficoll-Paque density centrifugation and 0.8% ammonium chloride red cell lysis. Percentage of viable cells was determined by trypan blue exclusion dye.

Cell labeling

Cells were labeled with Aldefluor reagent (Vancouver Canada) as described by the manufacturer. Cells were then stained with phycoerythrin (PE)–conjugated anti-CD34 (Miltenyi Biotec, Cologne, Germany) antibodies for 30 min at 4°C. Cells were washed and re-suspended in phosphate-buffered saline (PBS) with 2% fetal calf serum. Cells were then analyzed on coulter epics flow cytometer. Aldefluor reagent was excited at 488 nm. Gates were set up to exclude nonviable cells and debris. The negative fraction was determined using appropriate isotype controls (Figures 1 and 2). For consistent results, Aldefluor-stained cells must be analyzed within 2 h of labeling. However, cells retain their ability to convert the ALDH substrate for at least 24 h after collection. We stored cord blood samples overnight before Aldefluor labeling and analysis without any detectable effect on the ALDH profile. DEAB tubes or negative control tubes were done to confirm that cellular fluorescence was the result of the activity of cytosolic ALDH; cells were incubated with DEAB which is a specific, competitive inhibitor of cytosolic ALDH that is nontoxic to cells *in vitro* and *in vivo* for 15 min at 37°C. These steps were repeated for each sample to be tested.

Set-up analyzer

1. In set-up mode, a DEAB control sample was placed on the cytometer; on the FSC vs. SSC plot, the R1 region was adjusted to encompass the leukocyte population of interest based on scatter. 2. On the FL1 vs. SSC plot, the FL1 photo-multiplier tube (PMT)

Table 1. Count and percentage of CD34+ cells to the total leucocytic count (TLC) in the cord blood samples.

Parameter	Mean	Maximum	Minimum	Standard deviation
% of CD34+ cells to the TLC	3.60	8.10	1.40	1.40
Count of CD34+ cells × 103/µl	0.48	1.12	0.12	0.23

TLC: thin layer chromatography.

Table 2. Count of ALDHbr cells × 103/µl in cord blood samples.

Parameter	Mean	Maximum	Minimum	Standard deviation
Count of ALDHbr cells × 103/µl	0.29	0.89	0.05	0.19

Table 3. Comparison between means of CD34+ cells count and ALDHbr cells count in cord blood samples.

Cd34+cells count × 10^3/µl		ALDHbr cells count × 10^3/µl		t-test	Significance (2-tailed)
Range	M±SD	Range	M±SD		
0.12-1.16	0.49±0.23	0.05-0.89	0.29±0.23	14.07	0.000**

**Difference is statistically significant.

voltage was adjusted so that the right edge of the stained population was placed at the 2nd log decade on the dot plot. The tube was removed. All cells were fluorescent due to the intracellular substrate.
3. The corresponding ALDH test sample was placed on the cytometer. The R2 region was adjusted to encompass the cell population that was side scatter-low and ALDH-bright. The tube was removed.
4. For data acquisition of test samples, the analyzer was put in acquisition mode and 100,000 events were collected on each ALDH and DEAB sample using the same instrument settings. DEAB control regions might need to be adjusted for each sample. ALDH-bright, SSC-low stem cells appeared in the R2 region.
5. Analysis for double expression of ALDH bright cells and CD34 +ve cells was done.

Data handling

1. FSC vs. SSC dot plot and region R1 that would encompass the leukocyte population of interest based on scatter were created.
2. Two FL1 vs. SSC dot plots were created gated on R1. A region R2 was created in both plots that began at the 2nd log decade of FL1 and was within the range of 200-400 on side scatter.
3. An ALDH positive sample data file was opened. The R1 region was adjusted in FSC vs. SSC dot plot to encompass the "viable" leukocyte population.
4. On the FL1 vs. SSC dot plot, the R2 region was adjusted to encompass the SSC low, ALDH-bright cells
5. Using the corresponding DEAB control tube, placement of the R2 region on the ALDH sample was verified by making sure that there were few or no events in the R2 area.
6. Region statistics were added to the plots.
7. The percentage of the ALDH-bright population was found from the percentage gated in R2 from the FL1 vs. SSC dot plot.

Statistical analysis

Statistical package for the social sciences (SPSS version 10.0) software was used for data analysis. The Student's paired t-test for significance of no difference was used throughout this report.

RESULTS

The study population included 46 umbilical cord blood samples collected from immediate newborns, 22 males (47.8%) and 24 females (52.2%). The mean weight of the newborns was 3258.7 g, with a minimum of 2,300 g and a maximum of 3,900 g. The mean white blood cells count in the tested cord blood samples was 13.6 × 10^3/µl, with a range of 5.7 × 10^3/µl to 27.3 × 10^3/µl, while the mean blood hemoglobin of the tested cord blood samples was 14.5 g/dl, with a range of 10.6 to 17.2 g/dl.

The mean percentage of the CD34 cells to the total leucocytic count was 3.6%; with a minimum of 1.4% and a maximum of 8.1% (Table 1); while the mean count of the CD34 cells × 10^3/µl in cord blood samples was 0.48 × 10^3/µl with a minimum of 0.12 and a maximum of 1.16 × 10^3/µl (Table 1). The mean count of the cells expressing the ALDH enzyme × 10^3/µl in cord blood samples was 0.29 × 10^3/µl, with a minimum of 0.05 and a maximum of 0.89 × 10^3/µl (Table 2). We found a statistically significant difference when we compared between the mean count of CD34+ cells and the mean count of ALDH expressing cells (Table 3). Also, there was a significant positive correlation between the count of CD34+ cells and the count of ALDH expressing cells (Figure 2). The mean percentage of ALDH enzyme expression among the CD34+ cells in the cord blood samples was 61.3% with a minimum of 28% and a maximum of 94.6% (Table 4 and Figure 3). Significant correlations were found between the WBCs count in the cord blood samples and both the

Table 4. Percentage of ALDHbr cells to the $CD34^+$ cells in cord blood samples.

Parameter	Mean	Maximum	Minimum	Standard deviation
% of ALDHbr cells to CD34+ cells	61.30	94.60	28	19.80

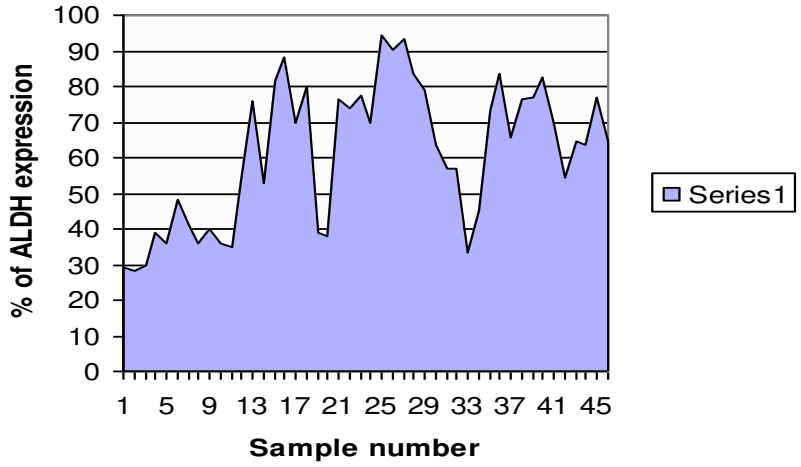

Figure 1. Percentage of ALDHbr cells to the CD34+ cells in cord blood samples

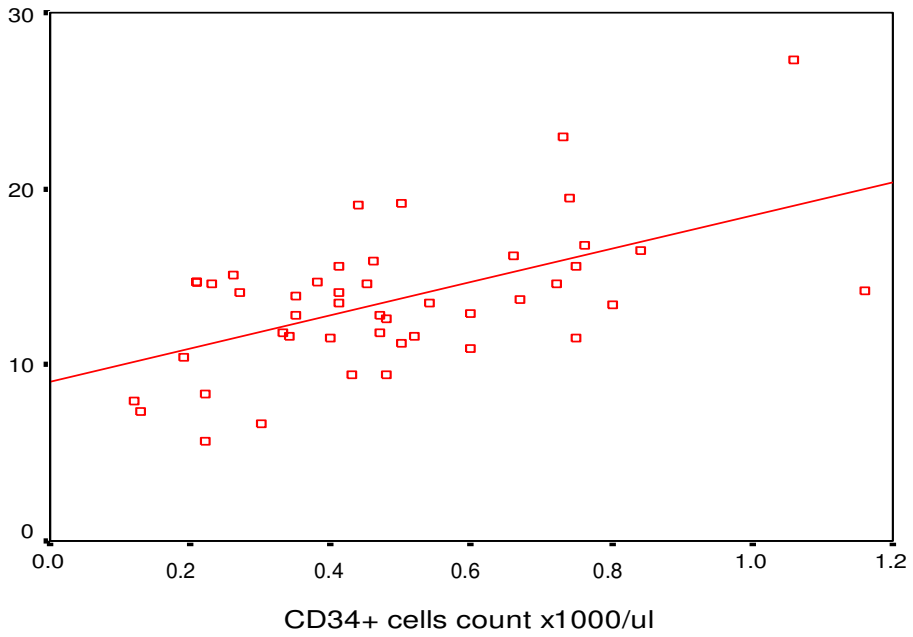

Figure 2. Correlation between WBCs count and CD34+ cells count. Pearson correlation: 0.557. Significance: 0.000; correlation is significant.

CD34+ cell count and the count of ALDH expressing cells (Figures 4 and 5). No correlation was found between the sexes or the weight of the newborn and neither the CD34+ cells count nor the ALDH expressing cells count in the cord blood samples.

DISCUSSION

Human cord blood hematopoietic cells with high ALDH activity are highly enriched for primitive CD34+ cells and depleted for lineage-positive (Lin+) cells (CD3, CD14,

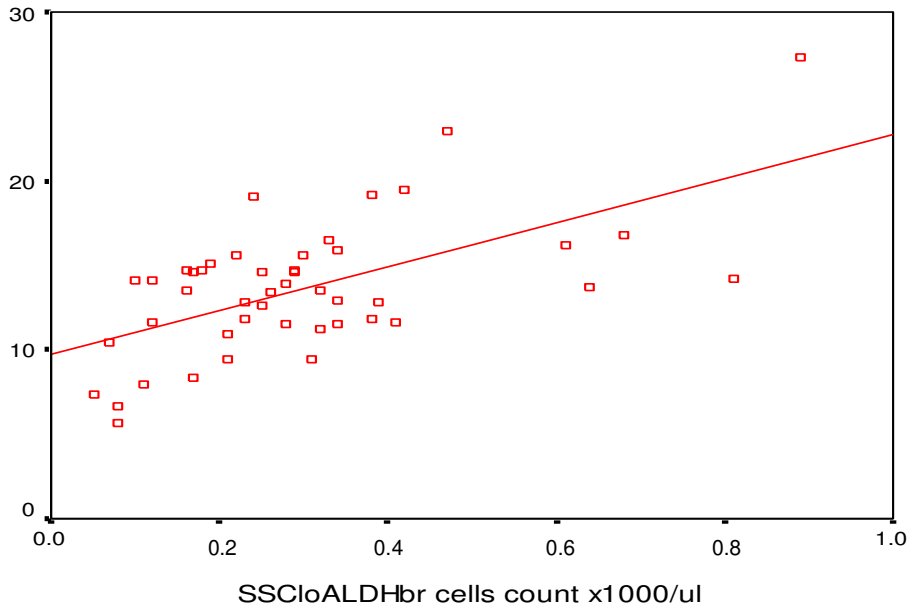

Figure 3. Correlation between WBCs count and ALDHbr cells count. Pearson correlation: 0.602. Significance: 0.000; SSClo ALDHbr: cells with low side scatter and bright ALDH. Correlation is significant.

CD20, and CD56), indicating that they do indeed represent a primitive hematopoietic cell population (Storms et al., 1999).

Methods to safely identify primitive HSCs with enhanced repopulating function are constantly sought for clinical stem cell transplantation. Conventionally, HSCs are purified using a single isolation strategy, such as the selection of cells based on cell surface phenotype (CD34 expression) or efflux of metabolic markers such as Hoechst dye by membrane pumps (Civin et al., 1984; Bhatia et al., 1997a; Sharkis et al., 1997; Gallacher et al., 2000; Guenechea et al., 2001; De Wynter et al., 1998; Goodell et al., 1997; Handgretinger et al., 2003). However, cell phenotype, such as CD34 surface expression, can vary depending on micro-environmental factors or cellular activation (Dao et al., 2003; Hess et al., 2003) and clinical procedures are incompatible with the use of toxic or DNA-intercalating dyes. Nontoxic cell-sorting strategies based on conserved stem cell function, in combination with cell surface phenotype, are necessary for clinical cell purification and may be useful for the study of complex developmental processes such as self-renewal versus the sequential transition from primitive HSCs to restricted progenitors. Our laboratory and others have demonstrated that cells with high intracellular ALDH activity from human UCB comprise a heterogeneous population of clonogenic progenitors and are enriched for NOD/SCID repopulating cells (Storms et al., 1999; Fallon et al., 2003; Hess et al., 2004). This isolation strategy uses a nontoxic, fluorescent substrate of ALDH, safely and effectively labeling cells with ALDH activity for selection by flow cytometry.

Many studies are describing ALDH expression in human CB (Hess et al., 2004; Christ et al., 2007; Hess et al., 2006; Gentry et al., 2007), peripheral blood stem cells (Fallon et al., 2003) and even acute myeloid leukemia (AML) BM cells (Cheung et al., 2007) data aiming to characterize normal BM ALDH+ cells are still limited to a few recent reports (Pearce and Bonnet, 2007; Gentry et al., 2007). Our interest in ALDH detection relies on the consideration that FCM based ALDH activity assessment is exploited in order to evidence a conserved stem cell function, rather than to merely identify a stem cell antigen (Morita et al., 2003).

In the current study, only 61.3 ± 19.8% of CD34+ cells were found to express ALDH activity. Robert et al. (1999) found that 74% ± 20 of CD34+ cells express ALDH activity, while David et al. (2004) found that the percentage was 91 ± 1.4%. Christ et al. (2003) found that the percentage was 95 ± 1%. These data strongly suggest that there is functional heterogeneity within the CD34+ cell population and that further purification of human stem or progenitor cells may be achieved through the analysis of ALDH activity. ALDHbr UCB cells was found to be 2% of the thin layered chromatography (TLC) while it was about 1% in the studies done by Christ et al. (2003) and David et al. (2004).

In the current study, no relation could be found between sex or weight of the newborn and expression of ALDH or CD34+. It was reported that birth weight of the neonate did not affect the mono-nucleated cell count (MNC) and subsequently CD34+ cell count. Hiett et al.

(1995) reported that there was no significant difference in the mean number of progenitors/UCB unit according to newborn weight. On contrary to these results, Arovita et al. (2005) reported that the correlation between birth weight and CD34+ cell concentration was statistically clearly significant. In this study, they tested 1368 CB samples for associations of selected factors as birth weight. Another study included 3838 CB units analyzing CD34+ cell contents only on units with a volume > 80 ml, a correlation analysis of CD34+ count and weight, revealing that baby weight was associated with higher CD34+ cell content in UCB (P = 0.0001). In this same study, a correlation analysis of CD34+ count and sex revealed that male newborns was associated with higher content of CD34+ cells (P = 0) (Guenechea et al., 2000).

Arovita et al. (2005) reported also that male infants had significantly higher median CD34+ cell concentration than female infants (31.8 × 103/µl vs. 30.2 × 103/µl, respectively (P = 0.03). There are several potential applications to this strategy for identifying and isolating HSCs. Enumerating ALDHbr cells may be a more reliable means for quantitating the transplantable stem cells in bone marrow, peripheral blood and UCB. Isolating ALDHbr cells also may be an effective method for purging autologous bone marrow or peripheral blood stem cell collections of tumor cells (Colvin et al., 1999). According to the manufacturers, the Aldefluor kit is active against the ALDH-1 isoform but not the ALDH-3 isoform. Both ALDH1 and ALDH3 are reportedly involved in chemoresistance (Civin et al., 1984; Bhatia et al., 1998; Bhatia et al., 1997b). In this study, we confirmed the use of the ALDH substrate kit to identify cord blood stem/progenitor cells expressing CD 34 via multicolor flow cytometry of cord blood ALDH+ cells.

A study done by Schuurhuis et al. (2013) showed marked difference between ALDH activity of HSC and LSC with the AML BM indicating the importance of ALDH activity as a functional stem cell biomarker and its usefulness in identification and purification of HSC and LSC with the aim of treatment decision making, relapse prediction and development of LSC specific therapies. Although HSC and LSC can, in a considerable part of AML cases, be distinguished using aberrancies of marker expression (van Rhenen et al., 2007a; van Rhenen et al., 2007b; Jordan et al., 2000) and scatter properties (Terwijn et al., 2007; Janssen et al., 2011), assessment of ALDH activity enables such discrimination in all AML cases even in the absence of aberrancies.

ALDH has received considerable attention as a functional marker for identification of cells with enhanced tumorigenic/metastatic potential and elevated therapeutic resistance in several cancers of epithelial origin (Ginestier et al., 2007; Jiang et al., 2009; Tanei et al., 2009). A possible application of ALDH detection by FCM to the field of acute leukemia may derive from the study of Cheung et al. (2007), in which the authors described ALDH expression in AML. They noted that in AML patients in complete remission, a relevant population of cells characterized by high ALDH activity remained (Cheung et al., 2007). So their data about multidimensional expression profile of ALDH combined with other hematopoietic antigens in normal BM precursors could represent the basis to distinguish by FCM leukemic from normal ALDH+ cells.

Overall, the ALDH kit is quick (1 h in total), easy to use and does not significantly affect cell viability or repopulation ability. The fluorescent substrate may be analyzed in conjunction with other common fluorochromes on a standard benchtop flow cytometer equipped with a 488 nm laser line. These properties suggest that this is a technique more suitable for the clinic than alternative techniques that are toxic and require expensive analytical equipment (for example, a UV laser) (Goodell et al., 1997).

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