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Concentration of the CDCP1 protein in human cord plasma may serve as a predictor of hematopoietic stem and progenitor cell content

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ABSTRACT

Successful hematopoietic stem and progenitor cell (HSPC) transplantation rests upon reliable methods for their enumeration in sources such as cord blood (CB). Methods used today are costly, time consuming and exhaust the limited number of cells needed for transplantation. The aim of this study was to analyze if surplus plasma from CB contains biomarkers that can predict HSPC content in CB. Frozen, surplus plasma from 95 CB units was divided into two groups based on CD34+ cell concentration. Birth weight, gestation age, gender, mode of delivery, collection volume, nucleated cell count and colony forming unit assay results were available. Samples were analyzed with a proximity ligation assay covering 92 different proteins. Two-group *t*-test with p-values adjusted for false discovery rate (FDR) identified 5 proteins that significantly differed between the two groups. CDCP1 was the most significant (FDR adjusted p-value 0.006). Correlation with CDCP1 concentration was most significant for CD34+ concentration and nucleated cell count. Multivariate analysis showed that CD34 and gender seemed to influence the level of CDCP1. In conclusion, CDCP1 was identified as a potential biomarker of HSPC content in CB. The finding also warrants further investigation for a possible role of CDCP1 in regulating HSPC presence in CB.

1. Introduction

Umbilical cord blood (CB) has become an established alternative cell source in hematopoietic stem cell transplantation (HSCT) besides bone marrow and mobilized peripheral blood. In later years, CB has also been used experimentally to treat infantile cerebral palsy, autism and other neurodegenerative disorders (Jensen, 2014; Lv et al., 2013). CB consists of the residual fetal blood left in the placenta. This blood carries hematopoietic stem and progenitor cells (HSPC) in the same concentration as adult bone marrow (Broxmeyer et al., 1989). This is in contrast to the low levels found in adult peripheral blood (Broxmeyer et al., 1989; Knudtzon, 1974). In the fetus, hematopoiesis is in a constant state of mobilization, starting in the yolk sac and aorta-gonad-mesonephros region before colonizing the liver, spleen and finally, following bone formation, bone-marrow at mid-gestation (Keller et al., 1999). The feto-maternal physiological processes governing these transitions and the rapid decline of HSPC in neonatal blood after birth are not well known. In adult hematopoiesis, however, the mechanisms of mobilization and homing of HSPC to bone marrow are well described and relates to hematopoietic growth factors, cytokines, chemokines, proteases and cell-cell interactions in bone marrow stroma (Hoggatt and Pelus, 2011). Some of these mediators are measurable in adult peripheral plasma (Szmigielska-Kaplon et al., 2015).

To retrieve as much HSPC as possible, CB is collected shortly after clamping of the cord. Maternal and neonatal factors such as ethnicity, gender, gestation age and mode of delivery, however, have been shown to influence the HSPC concentration in CB (Cairo et al., 2005; Aroviita et al., 2004a, 2005).

Successful HSCT rests upon reliable methods for HSPC enumeration. Frequently used methods to determine HSPC content are the colony forming unit (CFU) assay and quantification of CD34+ cells (Frandberg et al., 2016). However, these methods are both costly and time consuming and exhaust the limited number of cells needed for transplantation. CB collections are usually processed to a buffy coat before freezing to reduce volume and erythrocyte content, resulting in surplus plasma and erythrocytes. If analysis of factors in CB plasma

Abbreviations: CB, cord blood; HSCT, hematopoietic stem cell transplantation; HSPC, hematopoietic stem and progenitor cell; CFU, colony forming unit; CBBU, CB buffy coat; NC, nucleated cells; iniNC, initial CB collection; iniCD34, CD34+ cell concentration in initial CB collection; CBU, cord blood unit; CFU-GM, colony forming unit-granulocyte/monocyte colonies; PLA, proximity ligation assay; NPX, normalized protein expression; PCA, principal component analysis; FDR, false discovery rate.

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could be used instead of nucleated cells to predict HSPC content in CB, this would be a clear advantage.

The aim of the present study was to investigate whether surplus cord plasma from CB processing procedures contain biomarkers that correlate with the number of CFU and CD34+ cell content in the corresponding CB collection.

2. Material and methods

2.1. Collection of CB and analysis of CD34 and nucleated cell content

CB and clinical information on collection volume, birth weight, placental weight post collection, gestation week, gender and mode of delivery was obtained after parental informed consent as previously described (Frandberg et al., 2016). The study was ethically approved by the regional ethical review board, Gothenburg, Sweden. CB collections with total nucleated cell count ≥ $150 * 10^7$ were processed to a CB buffy coat (CBBU) using the Sepax system (Biosafe, Eysins, Switzerland) (Frandberg et al., 2016). The concentration of nucleated cells (NC) was measured for each initial CB collection (iniNC) and CBBU using a cell counter (Celldyn Sapphire, Abbott diagnostics, Santa Clara, CA, USA) and for each CBBU viable CD34+ cells concentration was measured using a Trucount tube-based CD34+ cell enumeration assay on a BD FACSCanto II (Becton Dickinson, San Jose, CA, USA) according to ISHAGE guidelines (Whitby et al., 2012). The viable CD34+ cell concentration in the initial CB collection (iniCD34) was calculated from the volume of the initial CB collection and corrected for NC recovery for each separate processing procedure. Resulting CBBU was finally supplemented with 10% DMSO and 1 ml was sampled into a separate tube and kept together with the corresponding full CB unit (CBU) in liquid nitrogen (Frandberg et al., 2016). Surplus cord plasma from processing was aliquoted and stored in −80 °C until analysis.

2.2. CFU assay

CBBU from the separately frozen tube was thawed and used for the 14-day CFU assay as described previously (Frandberg et al., 2016). The total number of CFU colonies (CFU-total) and CFU-granulocyte/monocyte colonies (CFU-GM) were counted manually under the microscope and the number of growing CFU-total or CFU-GM colonies per 100,000 seeded nucleated cells were calculated.

2.3. Proximity ligation assay

Frozen, surplus cord plasma was chosen from all CBU (n = 200) donated in 2013–2014 with available cord plasma samples. Based on iniCD34, 95 CBU:s with the highest and lowest results respectively, were chosen for further analysis. Accordingly, units with iniCD34 ≥ 50 cells/μl were considered high in HSPC content (CBU-H, n = 47) and iniCD34 ≤ 40 cells/μl low in HSPC content (CBU-L, n = 48). Units with iniCD34 between 40 and 50 cells/μl, *i.e.* intermediate in HSPC content, were not included in the study. CBU plasma samples were analyzed with proximity ligation assay (PLA) using the Proseek Multiplex Inflammation panel (Olink Proteomics, Uppsala, Sweden) covering 92 unique protein biomarkers. The assay yields semi-quantitative protein expression data on all individual biomarkers normalized to total protein expression (NPX) through real-time PCR using the BioMark HD platform (Fludigm, San Francisco, CA, USA) as described elsewhere (Assarsson et al., 2014).

2.4. Statistical analysis

Calculations were performed using SPSS version 22 (IBM, Armonk, NY, USA) and R (www.r-project.org). *t*-Test with false discovery rate (FDR) correction was used for comparison between groups and Spearman's rho for univariate analysis of correlations. Results were considered statistically significant if $p < 0.05$.

We used ordinary multiple linear regression to identify variables that made an important contribution to the variability of CDCP1 in plasma samples and to adjust for confounding variables. The multiple regression model was built using a so called purposeful stepwise approach where all secondary predictors and possible confounders were added one by one to the base model. Only variables with significance ≤ $p = 0.1$ were kept in the final model.

3. Results

3.1. Five proteins differed significantly between CBU high and low in CD34

To search for biomarkers in surplus cord plasma corresponding to HSPC content, a screening approach with a semi-quantitative array covering 92 unique proteins was used. There were 69 proteins (75%) detected in all 95 samples analyzed. An overall increase in the protein levels were seen in the CBU-H group ($p < 0.001$). Furthermore, five proteins that significantly differed between the CBU-L and CBU-H groups were identified; Caspase-8 (CASP8), C—C motif chemokine 23 (CCL23), Interleukin-18 receptor-1 (IL18R1), CUB-domain containing protein-1 (CDCP1) and T-cell surface glycoprotein CD6 isoform (CD6) (Table 1).

3.2. CDCP1 concentration correlated with both CD34 and CFU-GM in CB

Among the proteins identified, CDCP1 was the most significantly expressed. Correlation with CDCP1 concentration for each sample (n = 95) was then investigated. It was most significant for CDCP1 and iniCD34 (Spearman, $r = 0.54$, 2-tailed p-value < 0.0001), but also for CFU count (CFU-total; $r = 0.30$, p value 0.003 and CFU-GM; $r = 0.25$, 2-tailed p-value 0.02) and iniNC ($r = 0.36$, p-value < 0.0001). There was no correlation for CDCP1 with birth weight, gestation age or collection volume.

3.3. CD34 and gender influenced CDCP1 levels in CB plasma

To identify variables that made an important contribution to the variability of CDCP1 in plasma and to adjust for confounding variables, we used ordinary multiple linear regression. The multiple regression model was built with iniCD34 as primary predictor and collection volume, iniNC, birth weight of child, placental weight post collection, gestation age, gender and mode of delivery as secondary predictors or confounding variables. Descriptive statistics for all included variables is given in Table 2. IniCD34 and iniNC exhibited high-grade covariance ($r = 0.60$) and iniNC was omitted from the model. Using a stepwise approach, only gender and iniCD34 variables were significant ($p \leq 0.1$) and henceforth included in the final model. Under these conditions the β coefficient for iniCD34 gave an increase in CDCP1 of 0.02 NPX for each increase in iniCD34 (cells/μl). Female gender was associated with a higher baseline level of CDCP1 (Fig. 1) with a β coefficient of 0.49. Adjusted R^2 for the final model was 0.25, *i.e.* the model explains 25% of the variation in CDCP1 between samples. Intercept, β coefficients, confidence intervals and significance for all variables in the final model is

Table 1
Primary and FDR adjusted p-values for the five proteins that significantly differed between CBU-L and CBU-H groups.

Protein	p-Value	FDR adjusted p-value	Difference in NPX
CDCP1	8.88×10^{-5}	0.006	0.18
CD6	0.001	0.04	0.37
IL18R1	0.001	0.04	0.20
CCL23	0.002	0.04	0.30
CASP8	0.002	0.04	0.47

Table 2
Descriptive statistics for all clinical variables included in multiple regression analysis.

n = 95	Min	Max	Mean	SD
Gestation (days)	259	295	277	7.9
Birth weight (g)	2655	4625	3605	427.6
Placental weight(g)	405	808	582	93.5
Collected volume (ml)	78	179	127	23.3
iniNC*10^8/L	77.9	274.0	140.9	38.9
iniCD34 cells/μL	6.9	222.8	49.5	36.8

	Frequency	Percent
Gender		
Girl	48	50.5
Boy	47	49.5
Delivery mode		
No stress	62	65.3
Stress	33	34.7

given in Table 3. Check for model fit showed normally distributed residuals for CDCP1 NPX (data not shown).

Table 3
Intercept, β coefficients, significance and 95% confidence intervals for all variables in the final multiple regression model.

Model summary	β	Sig.	95% confidence interval	
Intercept	6.38	<0.0001	5.95	6.82
Girl	0.49	0.025	0.06	0.92
Boy	0	–	–	–
CD34	0.02	<0.0001	0.01	0.02

4. Discussion

HSPC concentration varies widely between CB collections and few qualify for CB banking in terms of total HSPC content (Querol et al., 2010). Aroviita et al. reported the median CD34+ cells concentration in 1368 full term CB collections to be 33 cells/μl, with a wide range of 1.9–663 cells/μl (Aroviita et al., 2004b) Differences most probably reflect on variations in hematopoietic physiology in late gestation and birth as part of mobilization and homing of HSPC to bone marrow from peripheral blood. Hence, gestation lengths, child gender, birth weight, mode of delivery and stress during delivery have all been shown to correlate with HSPC concentration in CB (Cairo et al., 2005; Aroviita et al., 2004a, 2005). Also, HSPC concentration in neonatal blood rapidly falls after birth. The percentage of CD34+ cells decline in median 25% in the first 3 h and reaches low adult levels in 60 h. The decline correlates with the level of erythropoietin (EPO) in cord and neonatal plasma after birth (Gonzalez et al., 2009) indicating the presence of factors that correlate with CD34+ cell concentration in CB. Also, HSPC secrete numerous inflammatory and regulatory factors when cultured *in vitro*, for example IL-8 and IL-16 (Majka et al., 2001), bringing forward the theory that HSPC concentration might influence cord protein composition directly. This is further strengthened by an increasing amount of publications showing general differences in protein composition comparing cord and adult plasma (Gonzalez et al., 2009; Castellano et al., 2017). Lastly, a recent study found a significant correlation ($r = 0.22$, $p = 0.05$) between levels of C—C chemokine ligand 28 (CCL 28) in cord plasma and CD34+ cell numbers in CB (Yoon et al., 2015) Taken together, our results add to the evidence that the protein profile in cord plasma correlates with HSPC concentration as measured through CD34+ cells concentration and CFU.

We implemented a screening approach, using a semi-quantitative array covering 92 unique proteins including hematopoietic growth factors, chemokines, cytokines, proteases and regulators of cell-cell interactions *i.e.* biomarkers of biological processes previously implied in homing and mobilization of adult HSC. With this method we identified five proteins that significantly differed between CBU high or low in HSPC concentration. The most significantly expressed protein between plasma samples with high and low concentration of HSPC was CDCP1. This is a cell surface transmembrane glycoprotein that was first described in 2001 as being overexpressed in colorectal cancer (Scherl-Mostageer et al., 2001) and has since then been implicated as a factor for poor prognosis in several other carcinomas (Ikeda et al., 2009; Awakura et al., 2008). CDCP1 has been proposed to play a role in regulation of cell differentiation and proliferation through interactions with extracellular matrix. CDCP1 is also a marker for HSPC and cells expressing CDCP1 can restore hematopoiesis in irradiated NOD/SCID mice (Conze et al., 2003). CDCP1 is co-expressed on CD34+ CD38- cells but not on mature hematopoietic cells in CB (Buhring et al., 2004).

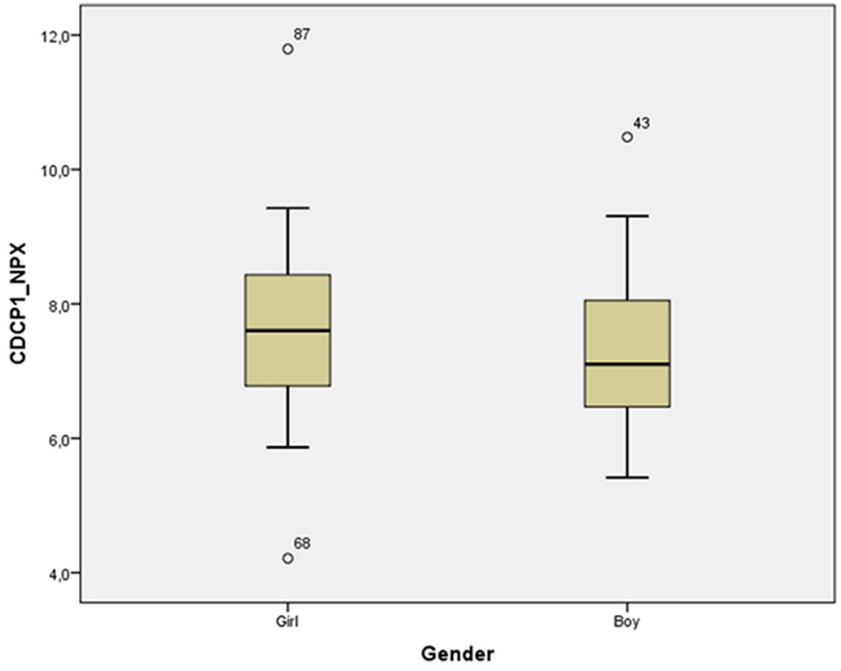

Fig. 1. CDCP1 normalized total protein expression (NPX) in girls and boys respectively.

Interestingly, CDCP1 gene expression has also been found to be upregulated in the EPCR expression subset of human cord blood with enhanced repopulation capacity (Fares et al., 2017).

Univariate analysis of our data shows that CDCP1 in CB plasma correlated most strongly with CD34+ cell concentration but also HSPC as measured by the functional cell cultivation based CFU assay, giving further support to this association. Finally, we built a multivariate regression model to further investigate the influence on CDCP1 concentration in plasma of birth and gestation related factors (birth weight of child, placental weight, gestation, and gender and delivery mode) against HSPC concentration related factors (iniNC and iniCD34). Collection volume was also included in the model to investigate collection related bias. Collection volume did not have a significant effect on CDCP1 concentration. This further supports a physiological relationship between CDCP1 and CD34+ cell concentration. As expected the concentration of nucleated cells and CD34+ cells exhibited covariance, and nucleated cell concentration could therefore be omitted from the model. Of the remaining variables only gender and CD34+ cell concentration were significant in the model. Female newborns seemed to have a slightly higher base level of CDCP1, which is evident in box-plots of CDCP1 for both genders (Fig. 1). Gender had a larger impact on CDCP1 (β coefficient $0.49 * 1$) when CD34+ cell concentration was in the low range. For high CD34 values the relative effect of gender was less substantial since the contribution of CD34 in the equation increased (β coefficient of 0.02* CD34 6.9–222.8 cells/μl). In our opinion, the model lends some support for a direct influence of CD34+ cell concentration on CDCP1 expression in plasma. However, CD34 cell concentration and gender taken together only explains 25% of the variation in CDCP1 so other factors must also be involved.

5. Conclusions

In conclusion, CDCP1 explains part of the variability in HSPC concentration in CB. The identification of CDCP1 is intriguing and warrants further investigation, both as its use as a biomarker for HSPC content weighted with other factors that correlate with CD34+ cells concentration in CB such as gender and TNC, but also for its role in HSPC migration and presence in cord blood.

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