Aldehyde dehydrogenase activity in cryopreserved cord blood cells for quality assessment prior to transplantation

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Abstract. In umbilical cord blood transplantation (UCBT), the number of cluster of differentiation (CD)34+ cells and colony-forming units (CFUs) in the cord blood (CB) graft positively correlate with patient survival. Therefore, these parameters are currently used for quality assessment of the cryopreserved CB cells in the attached segment that is considered representative of the CB in the main bag prior to UCBT. Since aldehyde dehydrogenase (ALDH) activity is high in hematopoietic stem cells, the number of ALDH-bright (ALDH+) cells was examined in comparison with the number of CD34+ cells and CFUs for the quality assessment of CB units. In the cryopreserved main bag, the number of ALDH+ cells in the CB unit exhibited positive correlation with the number of CD34+ cells, and with CFU-granulocytes/macrophages and total CFU counts. Furthermore, the concentration of ALDH+ cells in the cryopreserved attached segment was not significantly different compared to that of the main bag, suggesting that the attached segment is representative of the main bag. In conclusion, the present study suggested that ALDH+ cell counts in the cryopreserved attached segments may serve as a quality assessment indicator for CB units prior to UCBT.

Introduction

Umbilical cord blood transplantation (UCBT) is increasingly performed as an alternative treatment for hematological malignancies, including leukemia and lymphomas (1,2). Hematopoietic stem cells in cord blood (CB) are immature and limited in number, which may correlate to the relatively high incidence of engraftment failure and delays in UCBT (3). Hematopoietic stem cell activity is routinely estimated by flow cytometric detection of cluster of differentiation (CD)34+ cells, and by colony-forming unit (CFU) assays in which the ability of blood cells to form colonies in a semisolid medium is tested, for example, formation of granulocyte/macrophage (GM) colonies in the CFU-GM assay. Predictive markers of CB engraftment are important for successful UCBT, and reports suggest that increased numbers of total nucleated cells (TNC), CD34+ cells (3), and CFU-GM or total CFUs (4) in the CB graft are associated with better engraftment and survival in UCBT.

CB for transplantation is currently provided by the CB bank (CBB), where CB units (CBUs) are cryopreserved following harvesting during full term delivery. As cryopreservation causes a decrease in the absolute number of TNCs and CD34+ cells (5), a quality assessment following thawing is performed on selected CBUs (6). Because the cryopreserved CB in the main bag is shipped for transplantation, the CB in the attached segment provides the only source for indirect assessment of the CB quality in the man bag (7).

Aldehyde dehydrogenase (ALDH) is a cytosolic enzyme responsible for the oxidation of intracellular aldehydes. In an ALDH activity detection system (ALDEFLUOR kit) (8), ALDH converts fluorescently-labelled aminoaacetaldehyde, an ALDH substrate which freely diffuses into cells, into fluorescently-labelled aminoaacetate, a negatively charged product that is retained inside cells. Therefore, cells with high ALDH activity accumulate increasing amounts of the fluorescent aminoaacacetate and are subsequently detected as ALDH-bright (ALDH+) cells by flow cytometry (8). ALDH is considered to be a detoxifying enzyme that protects stem cells from cytotoxic effects, and its activity is reported to be high in primitive hematopoietic stem cells (8,9). Therefore, ALDH activity is considered to be a marker of stem cell activity. In peripheral blood stem cell (PBSC) transplantation, the number of ALDH+ cells in mobilized PBSC correlates with engraftment success following autologous transplantation (10). In UCBT, ALDH+ cells are correlated with CFU-GM, suggesting that ALDH activity may be suitable for quality assessment of CBUs (11).

In the present study, the association between TNCs, ALDH+ cells, CD34+ cells and CFUs was examined in 16 CBUs. In addition, the number of ALDH+ cells in the cryopreserved main bag were compared with those in the attached segment. The present results suggested that evaluation of ALDH activity...
in the cryopreserved CB cells of the attached segments may be useful for quality assessment prior to UCBT.

Materials and methods

CB collection and processing. Umbilical CB was collected into a 200 ml bag containing 28 ml citrate phosphate dextrose-adenine anticoagulant by venipuncture of the umbilical cord following full-term delivery, in cooperation with the Obstetric Clinic with Hyogo Cord Blood Bank (Nishinomiya, Japan). Informed consent was obtained from the mother to donate to the Cord Blood Unit (CBU) following CB transplantation or for medical research if not suitable for transplantation. CBUs that were not suitable for transplantation were selected for this study between November 2012 and December 2014. CB was mixed with 6% hydroxyethyl starch (Nipro Pharma Corporation, Osaka, Japan) at a ratio of 5:1 for 5 min, and centrifuged at 50 x g for 5 min at room temperature. The buffy coat layer was subsequently transferred to a new bag. The buffy coat was cryopreserved in 5% dimethyl-sulfoxide, 6% hydroxyethyl starch (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) and 4% human serum albumin (Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) in a final volume of 25 ml in the main bag, and ~0.4 ml in the attached tube which was divided into four attached segments (each volume was -0.125, 0.055, 0.11 and 0.11 ml) by heat sealing. CBUs were cryopreserved at -80˚C without rate-controlled freezing overnight (12), and were subsequently transferred and stored in liquid nitrogen. For quality assessment, main bags and attached segments were thawed in a 37˚C water bath, and TNC and CD34+ cell quantification, CFU assay and ALDH analysis were performed subsequently. The present study was approved by the institutional review board of Hyogo College of Medicine and Hyogo Cord Blood Bank (Nishinomiya, Japan).

Quantification of TNC and CD34+ cells. Quantification of TNCs was determined using an automated cell counter (XE-5000; Sysmex Corporation, Kobe, Japan). Quantification of CD34+ hematopoietic stem and progenitor cells was determined using a BD Stem Cell Enumeration kit (cat. no. 344563; BD Biosciences, San Jose, CA, USA), which included a fluorescein isothiocyanate-conjugated anti-CD45 monoclonal antibody (clone 2D1), a phycoerythrin-conjugated anti-CD34 antibody (clone 8G12) and 7-Amino-actinomycin D (7AAD). Cells at 2-20x10⁶/ml were incubated with these antibodies and 7AAD for 20 min at room temperature in the dark, then erythrocytes were lysed with ammonium chloride solution. Samples were analyzed using a FACSCanto™ II system and BD FACSCanto™ Clinical software version 2.4 (BD Biosciences). CD34+ cells were identified under sequential gating of the 7AAD- and CD45dim- cell populations according to the single platform guidelines of the International Society of Hematotherapy and Graft Engineering (13).

CFU assay. CFU assays were performed using a commercially available methylcellulose medium (MethoCult H4034 Optimum; Stemcell Technologies, Inc., Vancouver, BC, Canada) according to the manufacturer's protocol. Briefly, 2-4x10⁴ CB cells were cultured in semisolid methylcellulose medium containing recombinant human (rh) stem cell factor, rh granulocyte-macrophage colony stimulating factor (GM-CSF), rh G-CSF and rh interleukin-3 and rh erythropoietin in 35-mm petri dishes for 14 days at 37°C in a humidified atmosphere of 5% CO₂, CFU-GM, CFU-granulocyte/erythroid/macrophage/megakaryocyte (GEMM) and burst-forming units-erythroid (BFU-E) were identified by observation of GM, GEMM and erythroid colonies, respectively, using an inverted microscope as described previously (14). The number of each CFU was calculated as the mean number of colonies in three dishes. Total CFUs were calculated by summation of mean CFU-GM, BFU-E and CFU-GEMM values.

ALDH analysis. The ALDEFLUOR kit (Stemcell Technologies, Inc.) was used for detection of intracellular ALDH enzyme activity by flow cytometry, according to the manufacturer's protocol. Briefly, boron-dipyrromethene (BODIPY) fluorescent dye-labeled aminoacetaldehyde (BAAA) is a substrate for ALDH that diffuses freely into cells. This is converted by ALDH into BODIPY fluorescent dye-labeled aminoacetate (BAA), which remains trapped intracellularly, thereby emitting a green fluorescence that enables flow cytometric detection of ALDH+ cells (8). As a negative control, the background fluorescence of the ALDH- cells was obtained by inhibition of ALDH activity by diethylaminobenzaldehyde. Analysis of ALDH+ cells was performed using a FACSCanto II system.

Statistical analysis. Data are expressed as the mean ± standard deviation or as the median (range). Paired t-tests were used to assess differences between the main bag and the attached segment. Linear regression analysis was performed to assess the correlation between the two groups. All statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of thawed cord blood units. In UCBT, CD34+ cell counts and CFU counts in the CB graft are known to be good predictors for engraftment and patient survival. Therefore, in the present study, ALDH+ cell counts for UBC quality assessment were evaluated by comparison with these parameters. The number of TNCs, CD34+, and ALDH+ cells and total CFUs were analyzed in the CB from the main bags of 16 CBUs, and detailed results are provided in Table I. The number of TNCs and ALDH+ cells were 8.06±1.70x10⁶/CBU and 2.61±2.78x10⁵/CBU, respectively.

Association between ALDH+ cell count and CD34+ cells and total CFU counts. The association between TNCs, ALDH+ cells, CD34+ cells and CFUs was subsequently examined by linear regression analysis. The number of TNCs were not significantly correlated with the number of CD34+ cells (Pearson's correlation coefficient R=0.1458, P>0.05) or the number of ALDH+ cells (R=0.07254, P>0.05; data not shown). As presented in Fig. 1, the number of ALDH+ cells were significantly correlated with CD34+ cell counts (R=0.8686, P<0.0001) in post-thaw CB in the main bag. Next, three different colony-forming
Table I. Characteristics of thawed cord blood units in the main bags.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mean ± SD</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNCs (x10^6/unit)</td>
<td>8.06±1.70</td>
<td>7.76</td>
<td>5.50-12.25</td>
</tr>
<tr>
<td>CD34+ cells (x10^5/unit)</td>
<td>13.77±8.91</td>
<td>9.33</td>
<td>2.06-35.00</td>
</tr>
<tr>
<td>ALDH&lt;sup&gt;+&lt;/sup&gt; cells (x10^5/unit)</td>
<td>2.61±2.78</td>
<td>1.16</td>
<td>0.29-9.17</td>
</tr>
<tr>
<td>CFU-GM (x10^5/unit)</td>
<td>4.85±2.99</td>
<td>4.24</td>
<td>0.72-12.42</td>
</tr>
<tr>
<td>BFU-E (x10^5/unit)</td>
<td>3.18±1.98</td>
<td>3.37</td>
<td>0.72-7.69</td>
</tr>
<tr>
<td>CFU-GEMM (x10^5/unit)</td>
<td>0.04±0.14</td>
<td>0.14</td>
<td>0.0-0.54</td>
</tr>
<tr>
<td>Total CFUs (x10^5/unit)</td>
<td>8.70±4.56</td>
<td>6.82</td>
<td>1.44-17.46</td>
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</table>

Cryopreserved cord blood units in the main bags were thawed and analyzed (n=16). TNCs, total nucleated cells; ALDH<sup>+</sup>, aldehyde dehydrogenase-bright; CFU-GM, colony forming units-granulocyte/macrophage; BFU-E, burst-forming units-erythroid; GEMM, granulocyte/erythroid/macrophage/megakaryocyte; SD, standard variation; CD34, cluster of differentiation 34.

Comparison of ALDH<sup>+</sup> cells in the main bag and the attached segment. The concentration of ALDH<sup>+</sup> cells was subsequently evaluated in the main bags and attached segments of the CBUs. As presented Fig. 3A, the concentration of ALDH<sup>+</sup> cells in the main bag and in the attached segment was 10.05±1.76 and 9.06±1.76 cells/µl of CB, respectively, which was not significantly different (P=0.1731). Furthermore, the number of ALDH<sup>+</sup> cells exhibited a high linear correlation between the main bag and attached segments in the 16 CBUs tested in the present study (R=0.7224, P=0.0016; Fig. 2D). The significant correlation of ALDH<sup>+</sup> cell count with CD34<sup>+</sup> cell and total CFU counts supports the hypothesis that measuring ALDH<sup>+</sup> cell numbers may serve as an indicator of CB quality following cryopreservation.

Discussion

Umbilical CB cells are an alternative source of hematopoietic stem cells for transplantation. TNC, CD34<sup>+</sup> cell and CFU counts in a CBU have been previously demonstrated to positively correlate with good engraftment rate and improved survival following CBT (3); therefore, these parameters have been adopted as essential criteria for assessing UCB quality prior to transplantation. In the present study, ALDH activity in the CB was examined in parallel with TNC, CD34<sup>+</sup> cell and CFU counts, in order to evaluate it as a novel marker for UCB quality.

ALDH oxidizes intracellular aldehydes to carboxylic acid, and this process is involved in cellular detoxification, proliferation, differentiation and drug resistance (10). ALDH<sup>+</sup> cell presence is a novel marker for human hematopoietic stem cells (8). In the present study, the number of ALDH<sup>+</sup> cells was highly correlated with the number of CD34<sup>+</sup> cells. Storms et al (9) reported that the ALDH<sup>+</sup>/CD34<sup>+</sup> cell population contains primitive hematopoietic stem cells and progenitor cells. The same study additionally reported that ALDH<sup>+</sup>/CD34<sup>+</sup> cells do not contain primitive stem cells but contain a few progenitor cells, while ALDH<sup>+</sup>/CD34<sup>+</sup> cells exhibit no hematopoietic activity but contain erythroid cells (9). Thus, ALDH<sup>+</sup> cells represent primitive hematopoietic stem and committed progenitor cells. The results of the present study suggested that ALDH<sup>+</sup> cells may serve as a novel indicator of CB quality; however, whether ALDH<sup>+</sup> cells are more reliable indicators than CD34<sup>+</sup> cells requires further clarification.

Lee et al (11) reported that ALDH<sup>+</sup> cells are correlated with post-thaw CFU-GM counts in CB. The present study supported this finding and further demonstrated that ALDH<sup>+</sup> cells were correlated with BFU-E and total CFU counts. In particular, total CFU counts exhibited the strongest correlation coefficient among CFUs. Previous reports have used either CFU-GM (11) or total CFU (4) counts for assessment.
of hematopoietic activity in transplantation. The quality and quantity of mobilized PBSCs has been assessed by CFU-GM counts (15). In unpublished results from our group, CFU assay of mobilized PBSCs gave rise to >90% of CFU-GM colonies and only a few BFU-E and CFU-GEMM colonies (data not shown). This finding may explain why CFU-GM is commonly used for quality assessment of mobilized PBSCs in PBSC transplantation. By contrast, CB cells gave rise to approximately 40% of CFU-GM, 50% of BFU-E and 10% of CFU-GEMM colonies (data not shown). Therefore, CFU-GM represent <50% of total CFUs and the proportion of CFU-GM and non-CFU-GM (i.e., the sum of BFU-E and CFU-GEMM) differs considerably among individuals, indicating that CFU-GM may not be a suitable indicator of CFUs. Accordingly, in the present study, the number of ALDH<sup>br</sup> cells was better correlated with total CFUs than CFU-GM. Thus, total CFUs may be a better marker than CFU-GM in the quality assessment of CB.

Attached segments of CBUs are routinely used for quality assessment of the unit selected for transplantation. Rodríguez et al (7) demonstrated that there are no differences in the calculated number of TNCs, CD34<sup>+</sup> cells and CFUs between the main bag and the attached segment; thus, the attached segment is considered to be representative of the CBU in the main bag. However, another report demonstrated that the attached segment exhibited higher numbers of CD34<sup>+</sup> cells and CFU-GM than the main bag (16). Furthermore, the attached segment is much smaller in volume compared with the main bag, and extra caution is required in handling the attached segment to avoid a rise in temperature that may be...
harmful to CB cells. Therefore, a comparison of ALDH<sup>+</sup> cell concentration in the main bag and the attached segment was performed in the present study, to ascertain the suitability of the attached segment as representative for the main bag. The results demonstrated that the concentration of ALDH<sup>+</sup> cells in the attached segment was comparable to that in the main bag.

In conclusion, the present study examined the use of ALDH activity in the attached segments as a novel indicator of UBC quality assessment in the main bag prior to transplantation. Shoulars <i>et al</i> (17) have recently reported similar results that ALDH<sup>+</sup> cells in the segment are associated with CFUs and may be utilized for quality assays prior to shipping from a cord blood bank. Larger clinical studies will be required in the future to examine the correlation between the number of ALDH<sup>+</sup> cells and patient survival following UCBT.

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Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

J1 and TK performed the experiments using umbilical cord blood cells. SY analyzed and interpreted the data. SK and TK contributed for collecting and processing of umbilical cord blood. YF conceived and design the study and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the institutional review board of Hyogo College of Medicine and Hyogo Cord Blood Bank (Nishinomiya, Japan). Informed consent was obtained from the mother to donate cord blood to Hyogo Cord Blood Bank for CB transplantation or for medical research if not suitable for transplantation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References