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ORIGINAL ARTICLE

The impact of post-thaw colony-forming units-granulocyte/macrophage on engraftment following unrelated cord blood transplantation in pediatric recipients

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We retrospectively reviewed the engraftment kinetics following unrelated cord blood transplantation (CBT) in association with the post-thaw colony-forming units-granulocyte/macrophage (CFU-GM) number along with the numbers of total nucleated cells (TNC), $CD34^+$ cells and $CD3^+$ cells. A total of 71 cord blood units prepared for 53 patients (double-unit CBT in 18 patients) were evaluated. Either the number of infused CFU-GM or $CD34^+$ cells was significantly lower in patients who failed to achieve engraftment ($P = 0.028$ and 0.005, respectively). Post-thaw CFU-GM, TNC and $CD34^+$ cells correlated with the speed of neutrophil engraftment ($P = 0.004$, 0.037 and 0.004, respectively), whereas only CFU-GM showed a significant correlation with platelet engraftment ($r = -0.385$, $P = 0.024$). In double-unit transplants, the number of CFU-GM was the only significant factor predicting engraftment of the predominating unit ($P = 0.006$). We conclude that the post-thaw CFU-GM number is a reliable predictor of rapid engraftment after CBT as well as of the predominating unit in double-unit transplants. Thus, it would be important to perform post-thaw CFU-GM assay on cryopreserved aliquots from several candidate cord blood units in advance before CBT to avoid selecting the unit that might possess a low clonogenic potential.

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Introduction

Cord blood is widely accepted as a reasonable alternative to bone marrow as a source of hematopoietic stem cells in selected patients, provided that it has an adequate number of cells. The number of total nucleated cells (TNC) or $CD34^+$ cells has been commonly used to predict engraftment and successful outcomes not only after cord blood transplantation (CBT) but also after peripheral blood or marrow stem cell transplantation.[1–6] Although the content of colony-forming units-granulocyte/macrophage (CFU-GM) in the graft represents clonogenic potential, few studies have documented clearly the impact of post-thaw CFU-GM on engraftment in humans after CBT. The CFU-GM assay requires about 2 weeks of culture, and this time-consuming procedure might be less attractive for clinicians who want to know immediate results. However, because cord blood should be left frozen for a significant period before being thawed and infused, its functional quality after thawing cannot be guaranteed even though it had an adequate cell number before freezing. Recent studies indicate that functional $CD34^+$ cells can be reduced to a significant degree after a freezing/thawing process excluding cells positive for apoptotic markers.[7–9] It thus appears that the post-thaw functional clonogenic potential would be critical in clinical setting to predict engraftment after transplantation, especially after unrelated CBT that is associated with delayed engraftment and a higher rate of engraftment failure than unrelated bone marrow transplantation.[10]

In this study, we retrospectively analyzed the impact of post-thaw cell dose parameters, especially focused on the number of CFU-GM infused, on engraftment. Our hypothesis was that the number of infused CFU-GM might best predict the engraftment kinetics after CBT.

Materials and methods

Patients

Fifty-three children younger than 18 years of age underwent unrelated CBT between January 2003 and August

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Table 1 Characteristics of the patients

Characteristics	
Total no. of patients	53
Male/female	36/17
Median age, (years) (range)	6.1 (0.6–16.6)
Median weight, (kg) (range)	21.6 (8.1–72.0)
Diagnosis	
ALL/ABL	19
AML	19
JMML/CML	7
Marrow failure syndrome	5
Solid tumor	2
Adrenoleukodystrophy	1
Transplanted cord blood unit	
Single unit	35
Double unit	18
HLA disparity (A, B and DR)	
Single-unit transplantation	
0/6	3
1/6	29
2/6	3
Double-unit transplantation	
0/6 + 0/6	1
0/6 + 1/6	0
1/6 + 1/6	15
1/6 + 2/6	1
2/6 + 2/6	1
Conditioning regimen	
Myeloablative	48
Reduced intensity	5

Abbreviations: ALL = acute lymphoblastic leukemia; ABL = acute biphenotypic leukemia; AML = acute myeloid leukemia; JMML = juvenile myelomonocytic leukemias; CML = chronic myeloid leukemia; HLA = human leukocyte antigens.

2006 at Samsung Medical Center, Seoul, Korea. Patients' clinical data are shown in Table 1. Acute leukemia was the most frequent cause of transplantation ($n = 38$). All but five patients with marrow failure syndrome (three with severe aplastic anemia and two with Fanconi anemia) were conditioned with a myeloablative regimen. Eighteen patients received double units of cord blood to augment the cell dose. Thus, we evaluated a total of 71 cord blood units for CFU-GM assay as well as other cell dose parameters such as TNC, $CD34^+$ cells and $CD3^+$ cells using 1 ml of cord blood samples taken immediately after thawing just before transplantation. Written informed consents were obtained on every occasion.

Determination of cell doses and CFU-GM assay

To avoid cell clumping that could compromise accurate counting, 1 ml of thawed cord blood cells were immediately diluted by adding 49 ml of wash solution (minimum essential alpha-medium containing 10% fetal calf serum and 1% DNAse) before enumeration of CFU-GM, TNC, $CD34^+$ cells and $CD3^+$ cells. The number of TNC was counted using the automatic hematology analyzer Sysmex XE-2100 (TOA Medical Electronics, Kobe, Japan). The enumeration of $CD34^+$ cells was carried out by flow cytometry on the FACSort flow cytometer (Becton Dickinson, San Jose, CA, USA) using the BD Procount Progenitor Cell Enumeration kit (Becton Dickinson, San Jose, CA, USA), which is composed of triple fluorescence dye including nucleic acid dye (FL1), CD34-PE (FL2) and CD45-PerCP (FL3). The number of $CD3^+$ cells was also determined by flow cytometry as such population showing double positivity for CD3 and CD45. For CFU-GM assay, the cell suspension was centrifuged at 1500 r.p.m. for 10 min. After an additional course of washing, the cell count was performed to prepare 1 ml of 5×10^5 nucleated cell suspension, of which 0.3 ml (1.5×10^5 nucleated cells) was mixed with 3 ml of a progenitor assay medium (MethoCult GF H4544, StemCell Technologies, Vancouver, Canada) containing 1% methylcellulose in Iscove's modified Dulbecco's medium (IMDM), 30% fetal bovine serum, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml stem cell factor, 10 ng/ml granulocyte–macrophage colony-stimulating factor and 10 ng/ml interleukin-3. Finally, the samples containing 5×10^4 nucleated cells were plated in triplicate in 35×10 mm culture dishes. The dishes were incubated at 37°C in a humidified 5.5% CO_2 incubator for 14 days and colonies were counted using an inverted microscope.

Assessment of engraftment and chimerism status

Hematopoietic recovery was defined as the time point of the first 3 consecutive days with a count $\geqslant 0.5 \times 10^9$/l for neutrophils and of the first 7 consecutive days with $> 20 \times 10^9$/l for platelets without transfusional support. Chimerism status was evaluated from marrow aspirates by DNA fingerprint analyses, detected by polymerase chain reaction amplification of short tandem repeats regions at 1, 3, 6 months and 1 year after CBT. Engraftment required hematologic recovery from donor-origin cells. The cumulative incidence of engraftment was calculated by the Kaplan–Meier method. In double-unit transplantation, the 'winner' unit was determined by chimerism analyses as the predominating donor unit over the other 'loser' unit.

Statistics

The correlation between the cell dose parameters and the time to engraftment was assessed by the Pearson correlation analysis. To compare the difference in cell dose parameters between the group with engraftment and that with engraftment failure, a nonparametric comparison using Mann–Whitney *U*-test was carried out. Paired *t*-test was used for the comparison of the pre-freezing cell number with the post-thaw counterpart. To determine the factors predicting the predominating unit after double-unit CBT, the Wilcoxon signed-rank test was used. SPSS version 12.0 was used for all statistical analyses, and statistical significance was accepted when the $P < 0.05$.

Results

Recovery of TNC and $CD34^+$ cell dose after thawing

The numbers of TNC and/or $CD34^+$ cells that had been assayed by cord blood banks before freezing were

Table 2 Percent recovery after thawing and the infused cell dose

	% Recovery		*Infused cell dose*			
	TNC/kg (%, n = 71)	*CD34$^+$ cells/kg (%, n = 38)*	*CFU-GM (10^5/kg)*	*TNC (10^7/kg)*	*CD34$^+$ cells (10^5/kg)*	*CD3$^+$ cells (10^7/kg)*
Mean ± s.d.	83.4 ± 25.0	100.5 ± 57.4	1.64 ± 1.83	5.15 ± 2.50	1.93 ± 1.52	1.06 ± 0.96
Median (range)	80.1 (38.6–185.3)	93.0 (28.1–307.5)	1.01 (0–7.52)	7.70 (1.20–10.80)	1.35 (0.49–6.59)	0.69 (0.10–4.21)

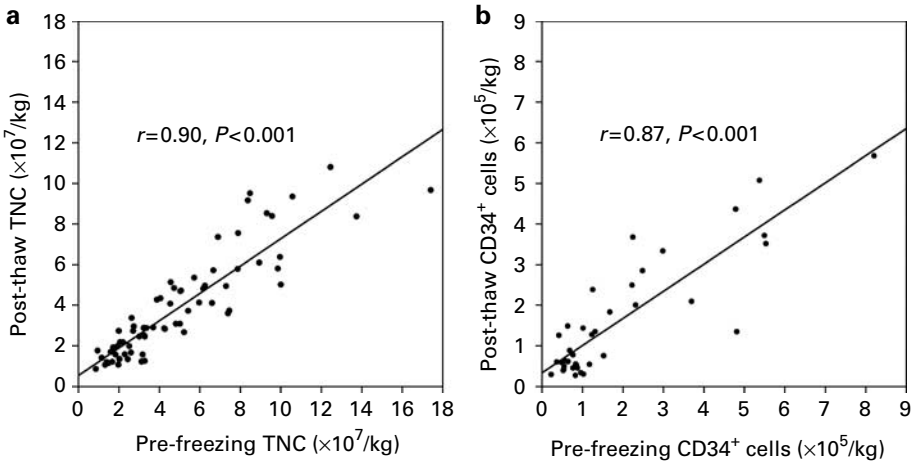

Figure 1 Correlation of the post-thaw cell numbers with the pre-freezing counterparts. (**a**) Post-thaw TNC numbers showed a good correlation with pre-freezing counterparts ($n = 71$; $r = 0.90$, $P < 0.001$). (**b**) Post-thaw CD34$^+$ cells showed a good correlation with pre-freezing counterparts ($n = 38$; $r = 0.87$, $P < 0.001$).

compared with those of post-thaw samples assayed at our institution (Table 2). The information about pre-freezing CD34$^+$ cell contents was available only for 38 units (53.5%). The pre-freezing and post-thaw numbers of TNC and CD34$^+$ cells showed a good correlation ($r = 0.897$, $P < 0.001$; $r = 0.870$, $P < 0.001$) (Figure 1). The recovery rates of TNC and CD34$^+$ cells (mean ± s.d.) were 83.4 ± 25.0 and 100.5 ± 57.4%, respectively. The median recovery was 80.1% (range, 38.6–185.3%) for TNC and 93.0% (range, 28.1–307.5%) for CD34$^+$ cells, respectively. The number of post-thaw TNC ($\times 10^7$/kg) was significantly lower than that of pre-cryopreservation ($P < 0.001$), whereas only a marginal difference was noted in CD34$^+$ cells ($\times 10^5$/kg) pre- and post-cryopreservation ($P = 0.08$) when assessed by paired *t*-test.

Correlation between cell dose parameters after thawing

When we compared the cell dose per unit from a total of 71 units, the number of CFU-GM correlated with each of TNC, CD34$^+$ cells and CD3$^+$ cells with a correlation coefficient of 0.556, 0.625 and 0.346, respectively. The strongest correlation was shown between TNC and CD34$^+$ cells ($r = 0.768$, $P < 0.001$).

Engraftment

The median time to engraftment was 17.5 days (range, 12–44 days) and 46 days (range, 14–148 days) for neutrophils and platelets, respectively. The cumulative incidence of neutrophil engraftment was 85.9% by day 60, and that of platelet engraftment was 70.6% by day 120. Excluding three patients who could not be assessed owing to premature deaths within 1 month post transplant, seven out of evaluable 50 recipients failed to obtain engraftment of donor-derived neutrophils. Either autologous recovery ($n = 4$) or primary engraftment failure ($n = 3$) occurred in four patients with acute leukemia, one with juvenile myelomonocytic leukemia, one with progressive adrenoleukodystrophy, and one with Fanconi anemia.

Cell doses and their influence on engraftment

The infused cell doses in 53 recipients are shown in Table 2. Post-thaw CFU-GM showed a wider range of distribution than the other parameters. Notably, CFU-GM did not grow post-thaw in two patients with acute leukemia even though the TNC data from the cord blood bank (8.93×10^7 and 5.97×10^7/kg) and those determined post-thaw at our institution (6.09×10^7 and 4.13×10^7/kg) were acceptable. Both of these patients eventually experienced engraftment failure. The post-thaw CD34$^+$ cell number was 0.49×10^5/kg for the first patient and was not available for the other. When the comparison was made between recipients who failed to show donor-derived hematopoiesis and those with successful engraftment, the numbers of infused CFU-GM and CD34$^+$ cells were significantly lower among those with engraftment failure ($P = 0.028$ and 0.005, respectively), whereas the numbers of infused TNC and CD3$^+$ cells did not show any difference ($P = 0.727$ and 0.976, respectively) between these two groups (Figure 2). Post-thaw CFU-GM,

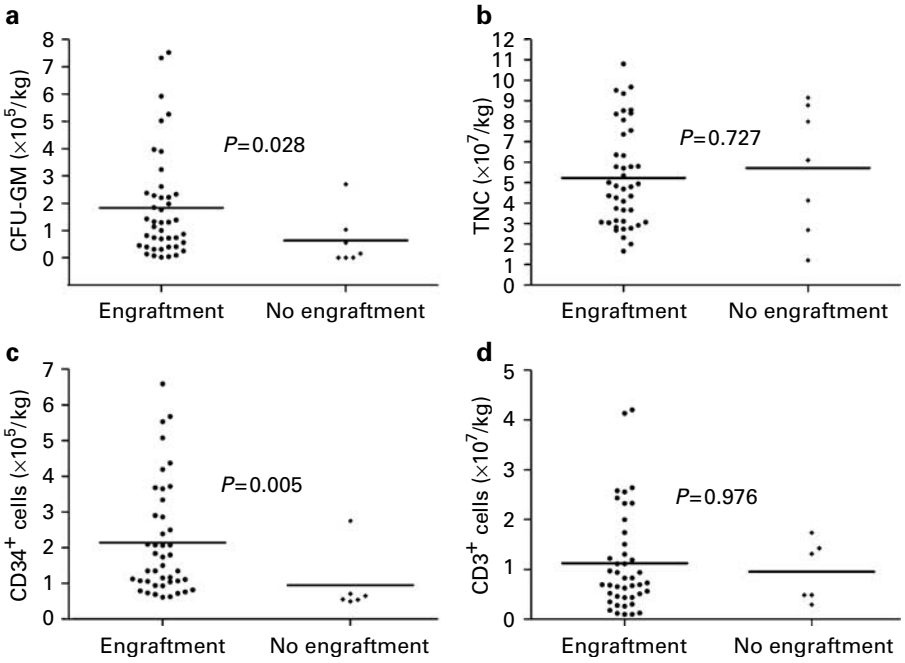

Figure 2 Difference in cell dose parameters according to the neutrophil engraftment. The infused CFU-GM and $CD34^+$ cell numbers were higher in patients who achieved engraftment (**a** and **c**), but the TNC and $CD3^+$ cell numbers were not significantly different between the two groups (**b** and **d**).

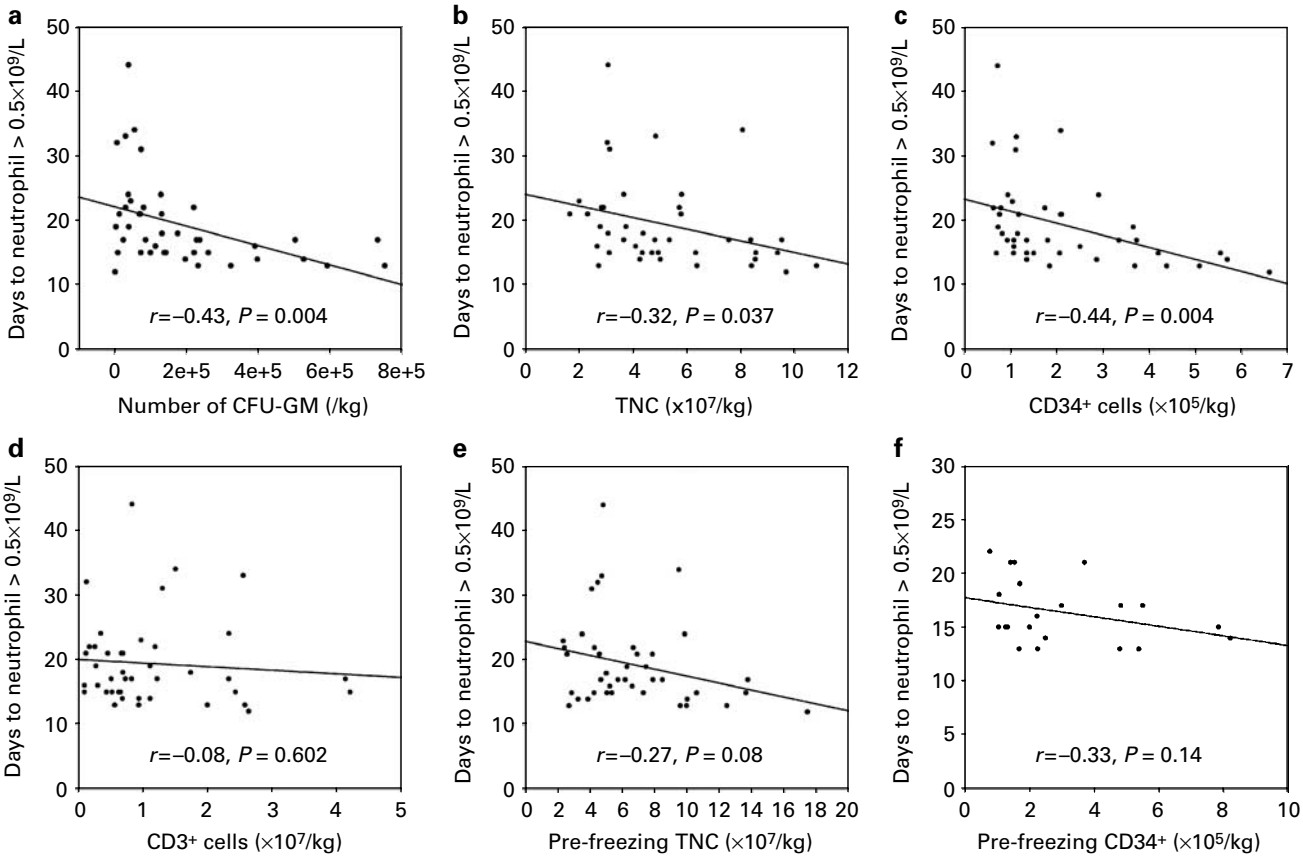

Figure 3 Correlation analyses between cell dose parameters and the time to engraftment. CFU-GM, TNC and $CD34^+$ cells counted after thawing (**a**–**c**) correlated with the time to neutrophil engraftment, whereas the number of post-thaw $CD3^+$ cells (**d**), pre-freezing TNC (**e**) and pre-freezing $CD34^+$ cells (**f**) showed no significant correlations with the time to neutrophil engraftment.

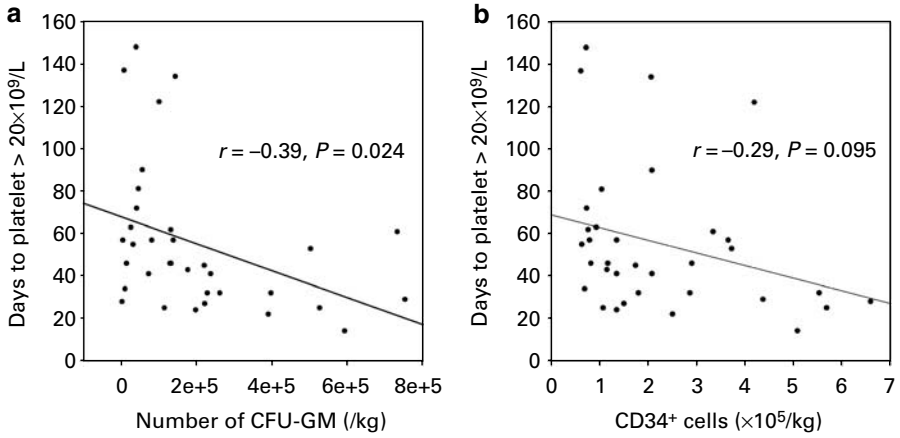

Figure 4 Correlation of infused CFU-GM and CD34$^+$ cells with the time to platelet engraftment. (**a**) A significant correlation was noted between the number of infused CFU-GM and the time to platelet engraftment ($r=-0.39$, $P=0.024$). (**b**) The number of infused CD34$^+$ cells showed no significant correlation with the time to platelet engraftment ($r=-0.29$, $P=0.095$).

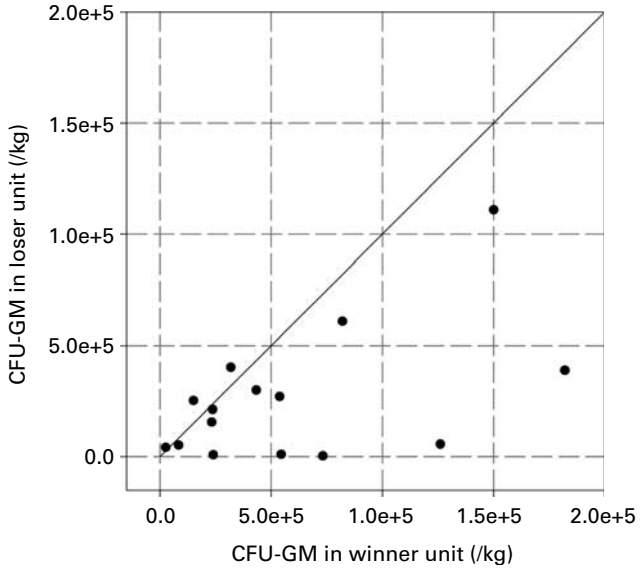

Figure 5 Comparison of the infused CFU-GM number of the predomination unit ('winner') versus that of the non-predominating unit ('loser') in double-unit CBT. The CFU-GM dose of the 'winner' unit was significantly greater than that of the 'loser' unit ($P=0.006$).

TNC and CD34$^+$ cells correlated with the time to engraftment of neutrophils ($P=0.004$, 0.037 and 0.004, respectively), whereas pre-freezing TNC and CD34$^+$ cells did not ($P=0.08$ and 0.14, respectively) (Figure 3). The number of infused CD34$^+$ cells did not correlate to the engraftment kinetics of platelets ($r=-0.29$, $P=0.095$), but CFU-GM showed a significant correlation ($r=-0.39$, $P=0.024$) (Figure 4). The number of TNC and CD3$^+$ cells also showed no correlation to platelet engraftment. In double-unit transplants, only the number of CFU-GM was significantly higher among the 'winner' units ($P=0.006$) (Figure 5).

Discussion

CBT usually gives rise to an inferior engraftment rate as well as slower engraftment kinetics than transplantation with bone marrow or peripheral blood stem cells when the recipient factors are similar. Because the cell dose is considered the most important factor affecting engraftment and survival after CBT if the human leukocyte antigen (HLA) disparity is acceptable, we tried to find out the impact of CFU-GM, which best represents the clonogenic potential but rarely used in CBT, along with TNC and CD34$^+$ cells on engraftment. Engraftment of allogeneic hematopoietic stem cells can be affected by a diversity of factors, which can be divided into either recipient or donor factors. Recipient factors that may affect engraftment include the type of recipient's disease, age, intensity of the conditioning regimen and so on. The HLA disparity and the difference in cell dose could be considered the most important variables affecting engraftment among the donor factors, provided the donor source is uniform.[11] Our study has limitations; in that the recipient's factors were variable owing to the heterogeneous patient population, but donor factors were relatively less diverse except for the cell dose in that the source of stem cells was invariably unrelated cord bloods and the HLA disparity to recipients was two or less per six antigens (83% of grafts were 1/6 Ag-mismatched), which is usually allowed for CBT.

Since about two decades ago, CFU-GM has been regarded as a useful parameter predicting engraftment of autologous bone marrow.[12–15] However, its clinical feasibility has much decreased nowadays in autologous peripheral blood stem cell transplantation in which CFU-GM cannot be used as a guideline for further rounds of leukapheresis because it is difficult to get timely results. Currently, CD34$^+$ cell enumeration has become a common practice not only in APBSCT but also in allogeneic blood or marrow transplantation owing to its convenience and rapidity as well as the good correlation to engraftment. Although the TNC count is still a part of the guideline for

selecting a cord blood unit, the number of $CD34^+$ cells rather than TNC seems to be a more reasonable guide for the selection of an appropriate unit, in that it is a more functional element as long as it is counted by a reliable method even though the standardization of $CD34^+$ cell enumeration has not been established yet and there may exist a significant laboratory-to-laboratory variability. Unfortunately, the biggest cord blood bank in Korea does not even count $CD34^+$ cells before freezing due to the cost, which makes clinicians feel unconfident when choosing the 'cell-rich' unit based only on the pre-freezing TNC. Indeed, we observed two patients whose cord bloods were selected according to the TNC doses without information about $CD34^+$ cell numbers, but proved to contain low $CD34^+$ cells and even no CFU-GM when tested after thawing. Both patients eventually experienced engraftment failure.

The parameters we took into account when choosing an appropriate unit for transplantation include HLA disparity, pre-freezing TNC number offered by the cord blood banks and also pre-freezing $CD34^+$ cell number, if available. In this study, the pre-freezing cell dose did not correlate with either neutrophil or platelet engraftment. Post-thaw TNC, $CD34^+$ cell and CFU-GM numbers intercorrelated, but the post-thaw CFU-GM number was the best predictor of the speed of engraftment of both neutrophils and platelets. In line with our observation, Migliaccio *et al*.[16] reported that CFC in cord blood grafts more accurately characterized the ability of the grafts to achieve neutrophil and platelet engraftment in a timely manner than the corresponding TNC counts. However, their study had limitations in that CFC numbers had been assayed before freezing. More recently, Iori *et al*.[17] investigated the prognostic factors in patients with high-risk leukemia undergoing unrelated CBT. Although none of the number of TNC, the $CD34^+$ cell dose or the CFU-GM infused was found to significantly affect hematopoietic recovery, they concluded that CFU-GM should be considered the main parameter in selecting cord blood units for transplant because post-thaw CFU-GM dose was the only factor significantly affecting event-free survival.

Since CFU-GM cultured from the day of transplantation cannot provide any role in promoting the rate or speed of engraftment, it would be advantageous to know it in advance before selecting a unit to be transplanted. If we had known the CFU-GM number in advance in the two patients whose colonies did not grow, we would not have chosen the units and could have avoided engraftment failure. Thus, we recommend that pre-freezing TNC should not be used as the sole guide for selecting an adequate unit and that performing $CD34^+$ cell enumeration should be a prerequisite before cryopreservation in cord blood banks. In addition, we believe that if we knew the number of CFU-GM along with those of TNC and $CD34^+$ cells in several candidate units before transplantation, we could reduce the chance of choosing an inappropriate unit having an acceptable cell number but very low or even no clonogenic potential.

In conclusion, we believe that CFU-GM is one of the most useful parameters when selecting cord blood units for transplantation. In addition, we propose that cord blood banks should prepare and supply small aliquots of cryopreserved samples from several candidate units at least several weeks before transplantation to allow for the application of CFU-GM assay to select the best unit possible.

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