

ORIGINAL ARTICLE

Failure to achieve a threshold dose of CD34⁺CD110⁺ progenitor cells in the graft predicts delayed platelet engraftment after autologous stem cell transplantation

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In this study, we retrospectively analysed the utility of CD110 expression on CD34⁺ cells as a predictor of delayed platelet transfusion independence in 39 patients who underwent autologous peripheral blood stem cell transplantation. Absolute CD34⁺ cells and CD34⁺ subsets expressing CD110 were enumerated using flow cytometry. Of the 39 patients, 7 required 21 days or more to achieve platelet transfusion independence. Six of the seven patients received a dose of CD34⁺CD110⁺ cells below 6.0×10^4 /kg while 30 of 32 patients who achieved platelet transfusion independence in <21 days received a dose of CD34⁺CD110⁺ cells $>6.0 \times 10^4$ /kg ($P < 0.001$). Patients with delayed platelet engraftment received a median dose of 5.2×10^4 CD34⁺CD110⁺ cells/kg compared with a median dose of 16.4×10^4 cells/kg for those engrafting within 21 days ($P = 0.003$). Further analysis showed that $>6.0 \times 10^4$ CD34⁺CD110⁺ cells/kg was highly sensitive (93.8%) and highly specific (85.7%) for achieving platelet transfusion independence within 21 days. Delay in platelet transfusion independence translated into an increased requirement for platelet transfusion (median 6 vs 2 transfusions, $P < 0.0001$). The dose of CD34⁺/CD110⁺ cells/kg infused at time of transplantation appears to be an important factor identifying patients at risk of delayed platelet engraftment.

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Introduction

The speed of haemopoietic engraftment after autologous SCT is generally accepted to be a function of the number of stem cells infused at the time of graft administration. Rapid and consistent engraftment of all lineages is observed when a CD34⁺ cell dose over 10×10^6 /kg is given, but transplants utilizing this cell dose are a minority in clinical practice. At doses below this, engraftment is less reliable with one quarter of patients receiving $<10 \times 10^6$ /kg failing to achieve a platelet count of 20×10^9 /l by day 20 in one study¹ and an even higher percentage at the most common CD34⁺ cell doses infused of $1\text{--}5 \times 10^6$ cells/kg. Other factors associated with slower platelet engraftment include the use of BM rather than PBSCs and transplants performed for acute myeloid leukemia.² Delayed platelet engraftment (defined hereafter as an unsupported platelet count $<20 \times 10^9$ /l ≥ 21 days following autologous SCT) presents a particular clinical problem due to the associated requirement for supportive transfusion and the increased resource utilization associated with the effects of haemorrhage, the requirement for blood products and the morbidity associated with blood and platelet transfusion. Moreover, failure of platelet recovery by day 60 after autologous SCT is associated with a significant increase in the incidence of both relapse and non-relapse mortality.²

To date, no method has emerged for specifically predicting delayed platelet engraftment after SCT. A logical approach would be to analyse the number of platelet progenitor cells infused at the time of graft administration. However, accurate assessment of platelet or megakaryocyte progenitor cells has proved difficult since no specific phenotype for such cells exists. The thrombopoietin receptor c-mpl (CD110) is expressed on a subpopulation of CD34⁺ cells that includes but is not restricted to megakaryocyte progenitors.³ Binding of thrombopoietin to c-mpl induces megakaryocytic proliferation and differentiation.⁴ We analysed a series of patients undergoing autologous SCT to determine whether the dose of CD34⁺CD110⁺ cells infused would predict speed of platelet engraftment. We focused on autologous rather than allogeneic transplants to exclude the effects of factors not restricted to graft progenitor cell number (such as CMV infection, GVHD and myelosuppressive medications

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including methotrexate, ganciclovir and trimethoprim/sulphamethoxazole) on platelet recovery.

Materials and methods

Patients

We retrospectively analysed the graft composition of 39 patients who underwent autologous transplantation for various haematological and non-haematological diseases in the Blood and Marrow Transplant Unit at Westmead Hospital between March 2001 and July 2005 (Table 1). Institutional Ethics approval was given for the analysis. Patients were transplanted for the following diseases: non-Hodgkins lymphoma, $n=16$; multiple myeloma, $n=11$; Hodgkins lymphoma, $n=4$; Ewings sarcoma, $n=3$; neuroblastoma, $n=2$; amyloid, $n=1$; rhabdomyosarcoma, $n=1$; medulloblastoma, $n=1$.

Mobilization

PBSCs were mobilized with 4 g/m² CY and 10 µg/kg/day G-CSF for 4 days before initial stem cell harvest. Leucapheresis was performed on a Cobe Spectra using a 12l exchange according to standard methods. Collection on 1 day was performed in 34 patients; two consecutive collections were performed in five patients. One patient received BM stem cells in combination with PBSCs.

Immunophenotyping

Absolute CD34⁺ cells and CD34 subsets expressing c-mpl were enumerated using an in-house single platform viable CD34 flow cytometry assay.⁵ Briefly, for fresh samples 10 µl of haemopoietic progenitor cell (HPC) (obtained by reverse pipetting) product was stained with 5 µl of CD34 PE, 5 µl CD45 FITC, 5 µl CD110 APC and 10 µl of 7AAD in a TRUCOUNT tube (antibodies supplied by BD Bioscience (San Jose, CA, USA) were titred in-house using an HPC product) and incubated for 15 min at room temperature. There were no wash steps or lysis agent used in the procedure. A total of 450 µl of PBS was added to the tube immediately before analysis by flow cytometry. Initially, we used fresh HPCs to establish the coexpression of CD110 on CD34⁺ cells. To determine the cursor placement on our dot plots, we chose CD3 APC (same isotype as CD110) as a negative control. CD3 is not expressed on CD34⁺ cells but stains mature T cells within our samples giving rise to specific staining rather than non-specific staining, which is often experienced with an irrelevant isotype control. We found virtually no membrane staining of platelets using the CD110 antibody at the titration utilized in these experiments. For this study, the analysis was performed on cryopreserved samples. At thawing, a small pilot vial was removed from the liquid nitrogen storage container, immediately placed in a water bath at 37°C gently shaken and removed as soon as thawing was complete. A 10 µl aliquot was stained as described above. Post-thaw samples were not washed to remove DMSO nor diluted to achieve the desired cell count, to minimize potential errors. Data acquisition was performed within 30 min of thawing.

Flow cytometry

List mode data was acquired on a FACSCalibur flow cytometer and analysed using Cell Quest 3.1 software (BD Bioscience). A modification of the ISHAGE gating strategy described by Sutherland⁶ was used to obtain the absolute number of CD34⁺/CD110⁺ cells per microliter.

Gating strategy for the in-house no-lyse single-platform protocol

Figure 1 shows the gating strategy for CD34 analysis on a thawed PBSC product, using the in-house no-lysis protocol. FL1 threshold was set on CD45 expression and adjusted to minimize debris (Figure 1a). Viable leukocytes were identified as 7 AAD-negative events (Figure 1b). TRUCOUNT beads were enumerated in R6 (Figure 1f). Acquisition continued until a minimum of 100 CD34⁺CD110⁺ events were collected (this ensured the number of beads acquired always exceeded 3000). The number of CD34⁺CD110⁺ cells/µl was calculated as follows: no. of viable CD34⁺/CD110⁺ cells (Figure 1h) multiplied by the number of beads per TRUCOUNT tube divided by the number of beads counted (R6) multiplied by the sample volume.

Haemopoietic recovery after BMT

Autologous transplantation was performed following therapy using conditioning-chemotherapy regimens at standard doses (Table 1). Leukocyte engraftment was defined as the first of three consecutive days that neutrophils exceeded $0.5 \times 10^9/l$. Platelet engraftment was taken as the first day of platelet count $\geq 20 \times 10^9/l$ on at least three occasions, 7 days after the most recent platelet transfusion (as defined by the International Bone Marrow Transplant Registry). Platelet infusions were given as single units of pooled platelets. Infusions were given when platelet counts decreased below $15 \times 10^9/l$ or according to clinical needs determined by treating clinicians.

Statistical analysis

The statistical software packages SPSS for Windows Version 1.5 and Prism were used to analyse the data. Mann-Whitney and χ^2 -tests were used to compare distributions of continuous or biological variables by subgroups of interest. Two-tailed tests with a significance level of 5% were used throughout. Receiver operator characteristics (ROC)⁷ curves were used to illustrate the performance of CD110 in predicting time to engraftment (<21 vs >21 days).

Results

Details of cell numbers contained in autologous blood or marrow stem cell products

All patients undergoing autologous transplantation received at least 2×10^6 CD34⁺ cells/kg (Table 1). Median CD34⁺ cell number/kg infused was 3.9×10^6 (range: $2-27.4 \times 10^6/kg$). The median number of CD34⁺CD110⁺ cells/kg infused was $13.8 \times 10^4/kg$ (range: $2.3-130.3 \times 10^4/kg$). There was a weak correlation between the number

Table 1 Clinical details of 39 patients undergoing autologous stem cell transplantation

UPN ^a	Age (years)	Diagnosis	Conditioning	Auto-stem cell source (no. of harvests) ^b	CD34 ⁺ (kg × 10 ⁶ /kg)	CD34 ⁺ /CD110 ⁺ (× 10 ⁴ /kg)	Days to platelet (> 20 × 10 ⁹ /l)	Days to ANC > 0.5 × 10 ⁹ /l	No. platelet post-BMT ^c
Case 25	62	NHL	BEAM	PBSC (1)	27.4	130.3	8	7	2
Case 35	67	NHL	BEAM	PBSC (1)	4.3	36.5	10	8	1
Case 37	46	MM	HDM	PBSC (1)	8.2	55.0	10	14	0
Case 40	37	LPD	BEAM	PBSC (1)	10.5	118.0	10	10	2
Case 20	63	MM	HDM	PBSC (1)	2.5	3.5	11	17	0
Case 11	59	NHL	BEAM	PBSC (2)	2.2	8.8	12	9	0
Case 16	44	MM	HDM	PBSC (1)	2.0	6.3	12	19	0
Case 18	35	NHL	Thio/Bu/Cy	PBSC (1)	7.0	94.8	12	11	0
Case 17	35	NHL	BEAM	PBSC (1)	7.3	30.7	13	16	1
Case 5	46	MM	HDM	PBSC (1)	12.4	7.0	13	19	1
Case 31	54	MM	Flu/Mel	PBSC (1)	5.9	16.0	13	16	0
Case 1	56	NHL	BEAM	PBSC (1)	3.5	19.6	14	10	2
Case 2	41	NHL	Cy/TBI	PBSC (1)	2.0	7.9	14	21	1
Case 7	4	Nb	Carb/Etop/Mel	PBSC (1)	11.3	17.0	14	9	3
Case 21	2	Mb	Cis/Vin/Cy	PBSC (2)	3.3	7.9	14	12	2
Case 28	48	NHL	CBV	PBSC (1)	2.5	12.8	14	10	2
Case 29	31	ES	Bu/Mel	PBSC (1)	3.9	10.9	15	10	0
Case 19	51	MM	HDM	PBSC (1)	3.0	20.8	16	19	2
Case 22	61	NHL	BEAM	PBSC (1)	2.4	15.3	16	11	2
Case 4	32	HL	CBV	PBSC (1)	4.1	17.6	16	12	2
Case 14	39	MM	HDM	PBSC (1)	2.5	8.3	17	15	1
Case 27	44	HL	CBV	PBSC (1)	4.0	27.8	17	15	4
Case 34	58	NHL	BEAM	PBSC (2)	2.1	2.3	17	15	2
Case 39	44	HL	CBV	PBSC (1)	2.4	25.3	17	15	4
Case 41	55	amyloid	HDM	PBSC (1)	2.8	16.8	17	15	2
Case 10	10	NHL	TBI/Mel	PBSC (1)	7.0	17.0	18	23	5
Case 23	16	ES	Bu/Mel	PBSC (1)	6.4	36.0	18	12	3
Case 24	41	MM	HDM	PBSC (1)	2.1	10.3	18	15	1
Case 32	19	LPD	Thio/Bu/Cy	PBSC (1)	2.1	26.3	18	19	3
Case 33	64	MM	HDM	PBSC(1)	3.7	13.8	18	11	1
Case 38	65	MM	Mel	PBSC (1)	3.9	10.9	18	20	2
Case 13	9	ES	Bu/Mel	PBSC (1)	7.1	11.6	19	12	1
Case 6	14	HL	Etop/Cyt/Mel	PBSC (1)	3.5	5.4	21	13	5
Case 36	2	Nb	Mel/Carb/Etop	PBSC (1)	10.0	4.0	30	9	7
Case 3	46	MM	HDM	PBSC (1)	4.0	5.2	> 30	11	4
Case 12	3	Rh	Cy	PBSC (1)	6.3	3.8	31	24	4
Case 30	42	NHL	BEAM	PBSC (2)	2.1	5.1	31	21	4
Case 8	62	NHL	BEAM	PBSC (1)	6.3	43.5	39	12	9
Case 26	57	NHL	BEAM	PBSC (2)+ BM	2.4	5.7	> 60	11	16

Abbreviations: BEAM = carmustine, etoposide, cytosine arabinoside, melphalan; Bu/Mel = busulphan, high-dose melphalan; CBV = cyclophosphamide, carmustine, etoposide; Cis/Vin/Cy = cisplatin, vincristine, cyclophosphamide; ES = Ewing's sarcoma; HDM = high-dose melphalan; HL = Hodgkins lymphoma; Mb = medulloblastoma; MM = multiple myeloma; Nb = neuroblastoma; NHL = non-Hodgkins lymphoma; Rh = rhabdomyosarcoma.

Patients are presented in order of the rapidity of achieving platelet transfusion independence.

^aCases were numbered in order of presentation for transplantation.

^bNumber of stem cell harvests required to achieve adequate CD34⁺ cell number for transplantation.

^cNumber of occasions on which pooled platelets were given post transplant.

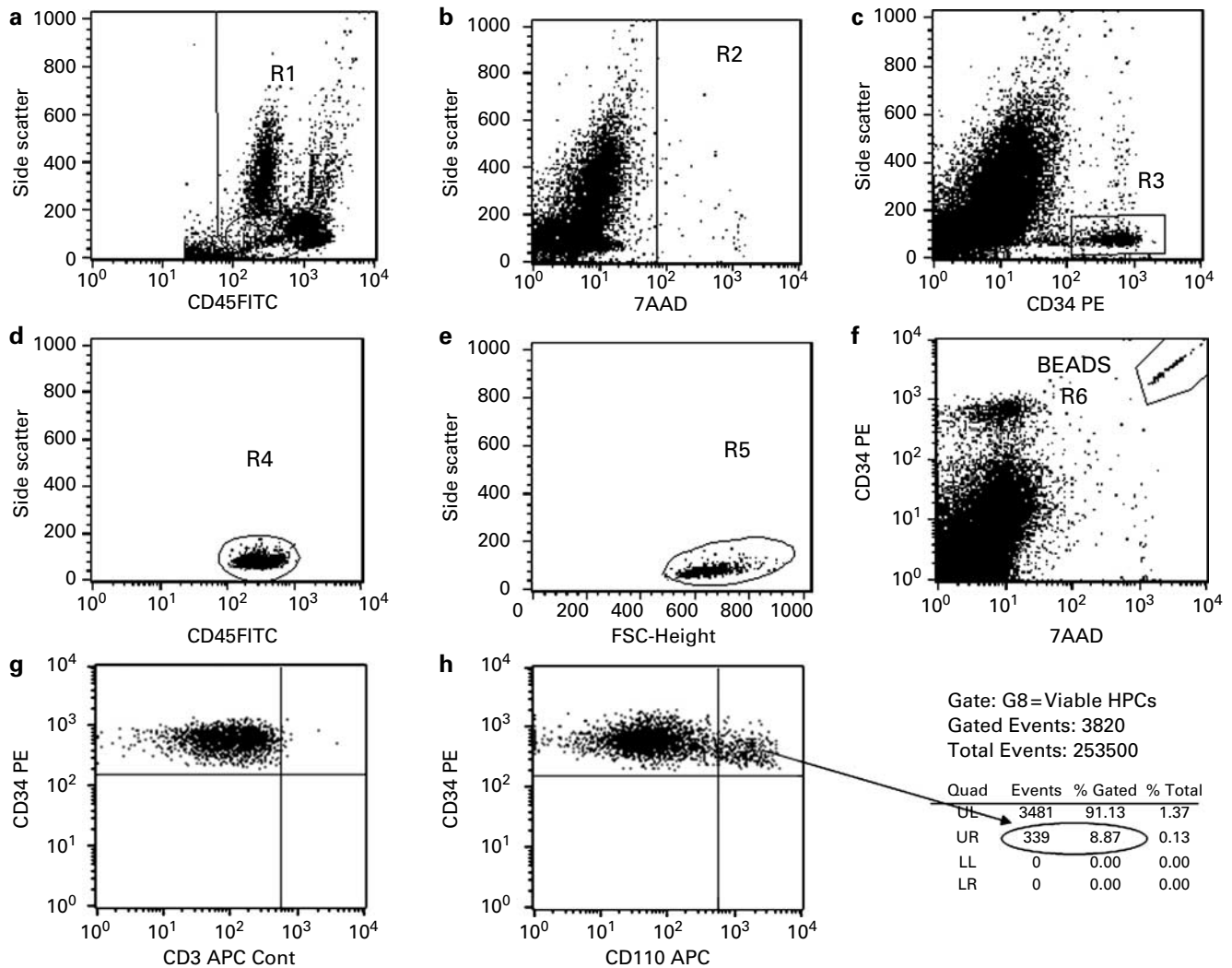


Figure 1 In-house no-lyse viable CD34 gating strategy. An example of a PBSCH product. Histogram (a) shows all CD45⁺ leukocytes region 1 (R1). Histogram (b) shows viable leukocytes gated on R1. Histogram (c) is gated on viable CD45⁺ events (that is, R1 and not R2) and CD34⁺ events are identified in R3. Histograms (d) and (e) are sequentially gated from R3 and R4 respectively. Histogram (h) shows the viable CD34⁺CD110⁺ events identified in R5. Histogram (f) shows ungated events and the bead count is obtained from R6. Histogram (g) shows control staining of cells in R5 with anti-CD3 antibody.

of CD34⁺ and the number of CD34⁺CD110⁺ cells contained within grafts, $r^2=0.48$ (Figure 2).

Platelet reconstitution post-autologous transplantation

Seven of 39 patients failed to achieve an unsupported platelet count of $>20 \times 10^9/l$ before day 21, achieving platelet independence between days 22 and >60 (Figure 3). Six of the seven received a graft containing $<6.0 \times 10^4$ CD34⁺CD110⁺ cells/kg. The remaining patient received a dose of 43×10^4 CD34⁺CD110⁺ cells/kg. He underwent autologous transplantation for a lymphoproliferative disorder associated with cold agglutinins with significant autoagglutination at room temperature that may have caused artefactual thrombocytopenia.⁸ Platelet engraftment occurred at day 39. Thirty-two patients became platelet transfusion independent within 21 days of transplantation. All but two received $>6.0 \times 10^4$

CD34⁺CD110⁺ cells/kg ($P<0.001$ compared with those showing delayed platelet transfusion independence). There was no obvious association between high doses of CD34⁺CD110⁺ cells/kg and rapidity of platelet engraftment (Figure 3). Two patients receiving $<6.0 \times 10^4$ CD34⁺CD110⁺ cells/kg displayed platelet engraftment within 21 days of transplantation but one of these two required over 6 months to sustain a platelet count greater than $100 \times 10^9/l$. A ROC curve was generated using the absolute number of CD34⁺CD110⁺ cells/kg for each patient, which showed that $>6.0 \times 10^4$ CD34⁺CD110⁺ cells/kg was both highly sensitive (93.8%) and highly specific (85.7%) for achieving platelet engraftment within 21 days (Figure 4).

Comparing patients displaying platelet engraftment within 21 days of transplant with those displaying engraftment beyond that time, the former group received a median of 16.4×10^4 CD34⁺CD110⁺ cells/kg

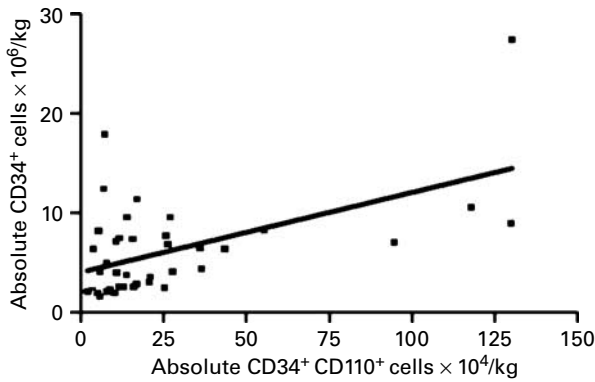


Figure 2 Correlation between absolute number of CD34⁺ and absolute number of CD34⁺CD110⁺ cells/kg infused at the time of transplant.

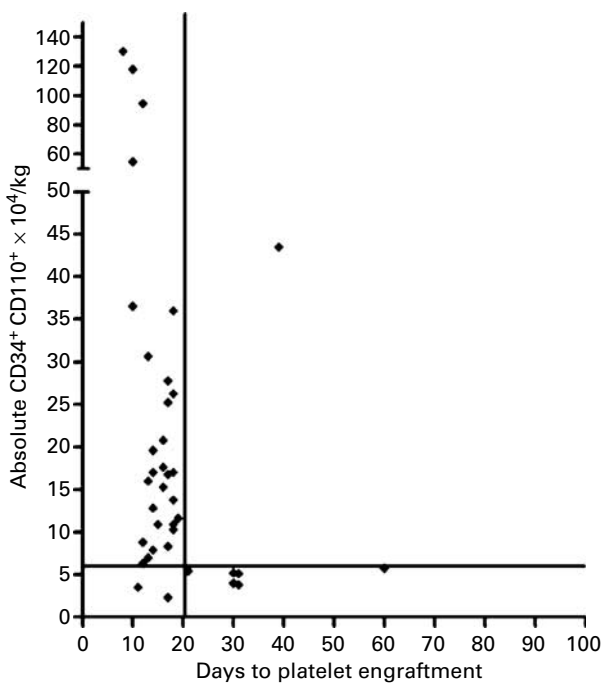


Figure 3 Number of days to platelet engraftment plotted against absolute number of CD34⁺CD110⁺ cells infused at the time of transplant. Each point represents one patient. Horizontal line indicates threshold value of 6.0×10^4 CD34⁺CD110⁺ cells/kg and vertical line 21 days post transplant.

compared with 5.2×10^4 CD34⁺CD110⁺ cells/kg for the latter group ($P=0.003$). This highly significant difference in absolute number of CD34⁺CD110⁺ cells infused was not observed when the percentage of CD34⁺ cells expressing CD110 was analysed (median 6.0 vs 5.5% for rapid vs slow platelet engrafters respectively, $P=0.45$). Three of the seven patients with delayed engraftment received CD34⁺ cell doses above 5×10^6 /kg. Patients with >21 days to platelet engraftment received platelet transfusions more often than those with <21 days to platelet engraftment (median 5 vs 2 transfusions, $P<0.0001$).

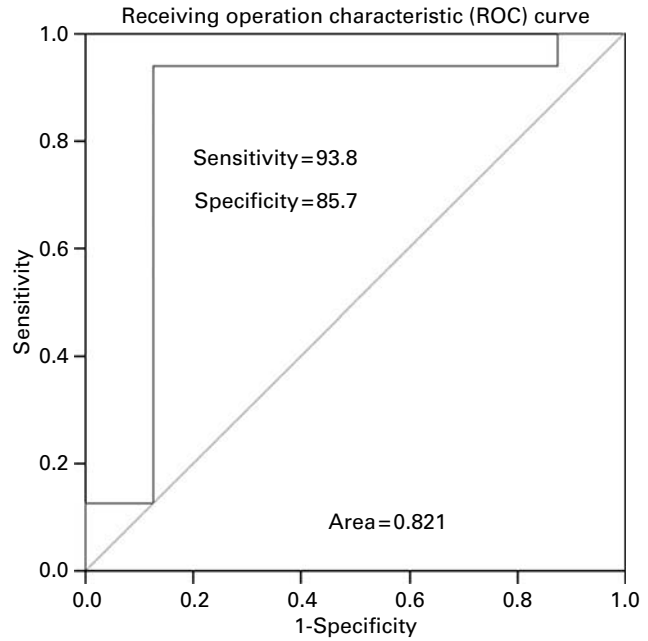


Figure 4 ROC analysis of the threshold dose of CD34⁺/CD110⁺ cells required for platelet engraftment within 21 days. More than 6.0×10^4 CD34⁺/CD110⁺ cells/kg gave the highest degree of sensitivity 93.8 and specificity 85.7.

Coexpression of CD110, CD41 and CD61 on CD34⁺ cells

Infusion of a large number of differentiated megakaryocyte progenitors might predict early but unsustained platelet engraftment post transplant. In an attempt to explain the rapid platelet engraftment observed in two patients with $<6 \times 10^4$ CD34⁺CD110⁺ cells/kg, we studied the coexpression of CD41 and CD61 on CD34⁺CD110⁺ cells and CD34⁺CD110⁻ cells. There was no difference in the percentage or absolute numbers of CD34⁺CD110⁺CD41/61⁺ cells or in the percentage or absolute numbers of CD34⁺CD110⁻CD41/61⁺ cells between the two patients with $<6 \times 10^4$ CD34⁺CD110⁺ cells/kg and two patients from this cohort receiving $>6 \times 10^4$ CD34⁺CD110⁺ cells/kg who engrafted at the same time (data not shown).

Discussion

Although the content of CD34⁺ cells in the graft is the single most important determinant of neutrophil and platelet engraftment after autologous blood or marrow transplant, many patients receiving a CD34⁺ cell dose widely considered adequate for transplantation ($>2 \times 10^6$ /kg) suffer delayed platelet engraftment. At CD34⁺ cell doses below 5×10^6 /kg, there is a 10–15% chance of delayed platelet transfusion independence, and even 5% of patients receiving $5\text{--}10 \times 10^6$ /kg CD34⁺ cells experience the same complication.⁹ Most patients undergoing autologous transplantation for haematological malignancy receive CD34⁺ cell doses in this range. In our series of 39 consecutive patients undergoing autologous transplant for haematological malignancy and solid tumours, over 60% received a CD34⁺ cell dose below 5×10^6 /kg while another

quarter received a CD34⁺ cell dose between 5 and 10 × 10⁶/kg. Only a minority (more than 10%) of patients in our series received a CD34⁺ cell dose above 10 × 10⁶/kg at which the risk of delayed platelet engraftment is negligible.

We analysed the correlation between recovery to platelet independence and the infused dose of cells coexpressing CD34 and CD110 (c-mpl, the thrombopoietin receptor). Our data show a strong correlation between infusion of a threshold dose (6 × 10⁴/kg) of CD34⁺CD110⁺ haemopoietic progenitor cells and achievement of platelet transfusion independence by day 21 post-transplant. Achievement of the threshold dose was a highly sensitive (93.8%) and highly specific (85.7%) test for rapid achievement of platelet transfusion independence following autologous transplantation. Patients receiving less than the threshold dose had a high chance of remaining platelet transfusion dependent beyond 21 days post transplant. For these patients, there was a significantly greater requirement for platelet transfusion. This measure has a superior correlation with platelet transfusion independence compared with the infused dose of CD34⁺ cells/kg. Indeed, three of seven patients in our series with delayed platelet transfusion independence received a CD34⁺ cell dose above 5 × 10⁶/kg. Above and below the threshold dose we identified, there was no correlation between the dose of CD34⁺CD110⁺ haemopoietic progenitor cells and the time to platelet transfusion independence. We could not identify over-representation of any specific disease state or conditioning regimen in the group of patients with delayed platelet transfusion independence.

Previous attempts to identify progenitor cell populations associated with platelet engraftment have focused on both immature CD34⁺ subsets lacking CD33 or CD38 and committed platelet progenitors expressing the platelet fibrinogen receptor gp IIb/IIIa (CD41/CD61). The number of CD34⁺CD33⁻ cells and the number of CD34⁺CD38⁻ cells infused at transplant do not correlate well with platelet transfusion independence.^{10,11} Dercksen¹² reported a threshold value of 0.45 × 10⁶ CD34⁺CD41⁺ cells/kg for rapid platelet engraftment following autologous transplant but half of the patients receiving less than this number also engrafted before day 21 limiting the value of this measure in clinical transplantation. Another study showed only weak correlation between the number of CD34⁺CD41a⁺ cells infused and time to platelet recovery to 20 × 10⁹/l¹³. Similarly, the number of CD34⁺CD61⁺ cells has not proved useful clinically in predicting slow platelet engraftment.^{1,14} When we analysed expression of CD41/CD61 in conjunction with CD34 and CD110, we could not identify a more accurate method of predicting platelet engraftment based on expression of more mature platelet markers.

In summary, we have identified a threshold dose for a progenitor cell subpopulation that predicts platelet transfusion independence within 21 days of autologous SCT in patients mobilized with CY and G-CSF. The assay is flow-based, can be easily incorporated into the enumeration of CD34⁺ cells at the time of harvest and gives rapid and reproducible results without the need for prolonged colony culture. Future studies will need to address whether exceeding the threshold value in poor mobilizers using

repeated collections or alternative mobilization strategies will allow for a directed approach to cell collection to avoid the morbidity and mortality associated with delayed platelet engraftment.

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