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Optimal Collection of Peripheral Blood Stem Cells By Assessing CD34$^+$ cells in Cancer Patients Administered with G-CSF after Chemotherapy

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ABSTRACT

The mobilization of stem cells from bone marrow to peripheral blood in cancer patients administered with recombinant human granulocyte colony-stimulating factor (G-CSF) were examined. Eight patients were injected with G-CSF subcutaneously at a dosage of 250 μg/body daily from WBC nadir by chemotherapy. The quantity of peripheral blood stem cells (PBSC) was assessed by the colony assay of CFU-GM, which peaked on the third to fifth day after the commencement of daily G-CSF administration. G-CSF significantly amplified the amount of PBSC in five of eight patients. These results suggest that a sufficient amount of stem cells for transplantation (1.7×10^5-5.4×10^5 CFU-GM/Kg) can be obtained in such cases if 5 L of blood was processed by leukapheresis. A flow cytometry analysis revealed that CD34$^+$ cells measured by mononuclear cell gating coincidentaly increased with the peak of CFU-GM and therefore their assessment may serve an useful index for rapid monitoring of PBSC. And, days 3-5 after the commencement of G-CSF injection may be the optimal timing for PBSC collection.

Key words: Peripheral blood stem cells, Granulocyte colny-stimulating factor, CFU-GM, CD34$^+$ cell, Flow cytometry.

INTRODUCTION

Hematopoietic progenitor cells are present in the peripheral blood of cancer patients during the hematological recovery period follwing myelosuppressive chemotherapy (2, 14, 17, 20, 24). By using these progenitor cells, the peripheral blood stem cell transplantation (PBSCT) has been carried out for bone marrow reconstruction instead of autologous bone marrow transplantation. PBSCT is known to bring about more rapid hematological recovery as compared with bone

marrow grafts and minimize the infectious risks during the agranulocyte stage (15, 21). It is also apparent that autologous PBSCT in advantageous over bone marrow harvest when patients have tumor invasion or radiation-induced hypocellularity in bone marrow (7, 15, 21, 22, 24).

As the collection of peripheral blood stem cell (PBSC) generally takes several days to obtain enough amounts of stem cells by leukapheresis, it is required to overcome this shortcomings. Recently, use of recombinant human granulocyte colony-stimulating factor (G-CSF) following myeloablative chemotherapy is reported to improve the recovery of PBSC (5, 11), although an appropriate timing for PBSC collection in not evaluated yet.

In the present study, we administered G-CSF to eight cancer patients who underwent chemotherapy to enhance PBSC, and analyzed the recovery and the optimal collection timing of PBSC by monitoring $CD34^+$ cell and CFU-GM.

PATIENTS AND METHODS

Patients

Eight patients included 4 cases of solid cancer (1 ovarian cancer, 1 choriocarcinoma, and 2 breast cancers) and 4 cases of hematological malignancy (1 Hodgikin's disease and 3 non-Hodgikin's lymphomas) (Table 1). The age distribution was from 26 to 75-years old. Clinical stages of 8 patients were various from 0 to IV. The performance status based on the criteria of WHO were between 0 and 2. One patient with breast cancer had the metastsis to lumbar

Table 1 *Characteristics of patients*

Case	Age (years)/Sex	Diagnosis	Clinical stage	Performance status[5]
1	41/F	Ovarian cancer	IV[1]	2
2	40/M	Choriocarcinoma	III[2]	1
3	35/F	Breast cancer	IV[3]	1
4	45/F	Breast cancer	0*	0
5	26/M	Hodgikin's disease	IA[4]	0
6	54/F	NHL	IA[4]	0
7	60/F	NHL	0*	0
8	75/M	NHL	IVB	0

Abbreviations: F; female, M; male, NHL; non-Hodgikin's lymphoma
[1]: TNM classification
[2]: TNM classification
[3]: TNM classification
[4]: Ann Arbor classification
[5]: WHO classification
*: Tumor had been removed by operation

vertebra and the bone marrow. Other patients had no apparent sign of metastasis to the bones and bone marrows.

Protocol of the trial

WBC after chemotherapy was counted daily by an automatic cell counter (NE-8000; Toa Medical Electronics Co., Kobe). G-CSF (Kirin Brewery Co., Tokyo) was subcutaneously injected daily at a dosage of 250 μg/body/day for successive 5 to 10 days after WBC became nadir by chemotherapy. Colony assay of CFU-GM and flow cytometry analysis of $CD34^+$ cell were also performed for every other day from the commencement of G-CSF injection (Fig. 1).

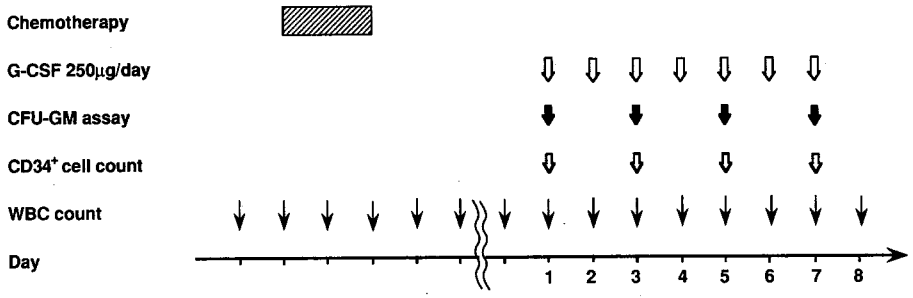

Fig. 1 Protocol of the trial.

Isolation of mononuclear cells

Every other day from the commencement of G-CSF injection, 10 mL of heparinized peripheral blood was collected. The mononuclear cell fraction was obtained by centrifugation in Ficoll-Isopaque gradient at 400 g for 30 minutes.

Colony assay of CFU-GM

Mononuclear cells (1×10^5/mL in a 35 mm-diameter plate) were suspended with α-minimum essential medium (α-MEM; GIBCO, Grand Island, NY) containing 0.8 % methyl cellulose (Katayama Chemical Industries Co., Osaka), 20 % fetal calf serum (Riggo Biochemical Co., Dubuque, Iowa), 100 U/mL interleukin 3 (IL3) (Genzyme Co., Boston, NY), and 100 ng/mL G-CSF (Kirin Brewery Co., Tokyo) (10) and were incubated in triplicate at 37℃ under 95% humdified air containing 5% O_2 and 5% CO_2. After 14 days of incubation, the number of CFU-GM composed of 40 or more cell aggregates was counted under the inverted microscope. The CFU-GM levels of peripheral blood at non-stimulated state by the present method were 4-15 colonies/1×10^5 mononuclear cells. This value is compatible to the result reported by McCarthy *et al* (9).

Flow cytometry

Cells expressing the surface CD34 antigen was identified by flow cytometry using indirect CD34 immunofluorescence. Fifty μL of heparinized blood was incubated with 50 μL solution of CD34 anti-HPCA-1 (My10) monoclonal antibody (mouse IgG1, Becton Dickinson Immunocytometry Systems., Mountain View, CA) for 30 minutes at 4℃, and cells were washed twice with 0.1% sodium azide in phosphate-buffered-saline (PBS), then incubated with 50 μL of FITC-conjugated goat anti-mouse IgG reagent (Ortho Diagnostic Systems Inc., Ralitan, NJ) for 30 minutes at 4℃. Erythrocytes were lysed with NH_4Cl buffer (NH_4Cl_3 8.29 g/L, $KHCO_3$1 g/L, 4×Na_2EDTA 0.037 g/L, pH 7.4) for 10 minutes at room temperature, and washed twice with 0.1% sodium azide in PBS. The lysate was analyzed by flow cytometry (ORTHO CYTORON ; Ortho Diagnostic Systems Inc., Raritan, NJ) equipped with the filter set for FITC-PE dual-color fluorescence. The gate was setted by a forward and side scattering cytogram to include mononuclear cell fraction because CD34 positive cells were exclusively present in this gate. Three thousand cells were aquired in list mode, and the frequency of the cells expressing CD34 antigen was calculated as percentage of all analyzed cells.

Statistical analysis

The relationship between the number of CFU-GM and WBC or $CD34^+$ cell was evaluated by a linear regression and correlation analysis using a commercially available computer program (SAS, SAS Institute Japan Inc., Tokyo). $P<0.01$ was accepted as statistically significant.

RESULTS

The serial change of blood cell count before and after G-CSF administration was shown in Fig. 2. WBC count increased to about 20,000/μL at maximum peaks in all cases. Five patients whose WBC count increased promptly (casel, 2, 6-8) displayed peak of CFU-GM at day 3-day 5, and their peak values of CFU-GM were more than 1,000/mL. On the other hand, three patients whose WBC count increased rather slowly (case3-5) required more than 9 days of G-CSF administration to attain WBC count 20,000/μL. The peak CFU-GM value of these three patients was less than 1,000/mL.

The peak of CFU-GM generally appeared earlier than that of WBC, and was coincident with that of $CD34^+$ cell in all eight cases.

To identify a valid and convenient parameter for monitoring of PBSC, the correlations between CFU-GM and WBC or $CD34^+$ cell were analyzed. As shown in Fig. 3, a positive correlation was obtained between the colony numbers

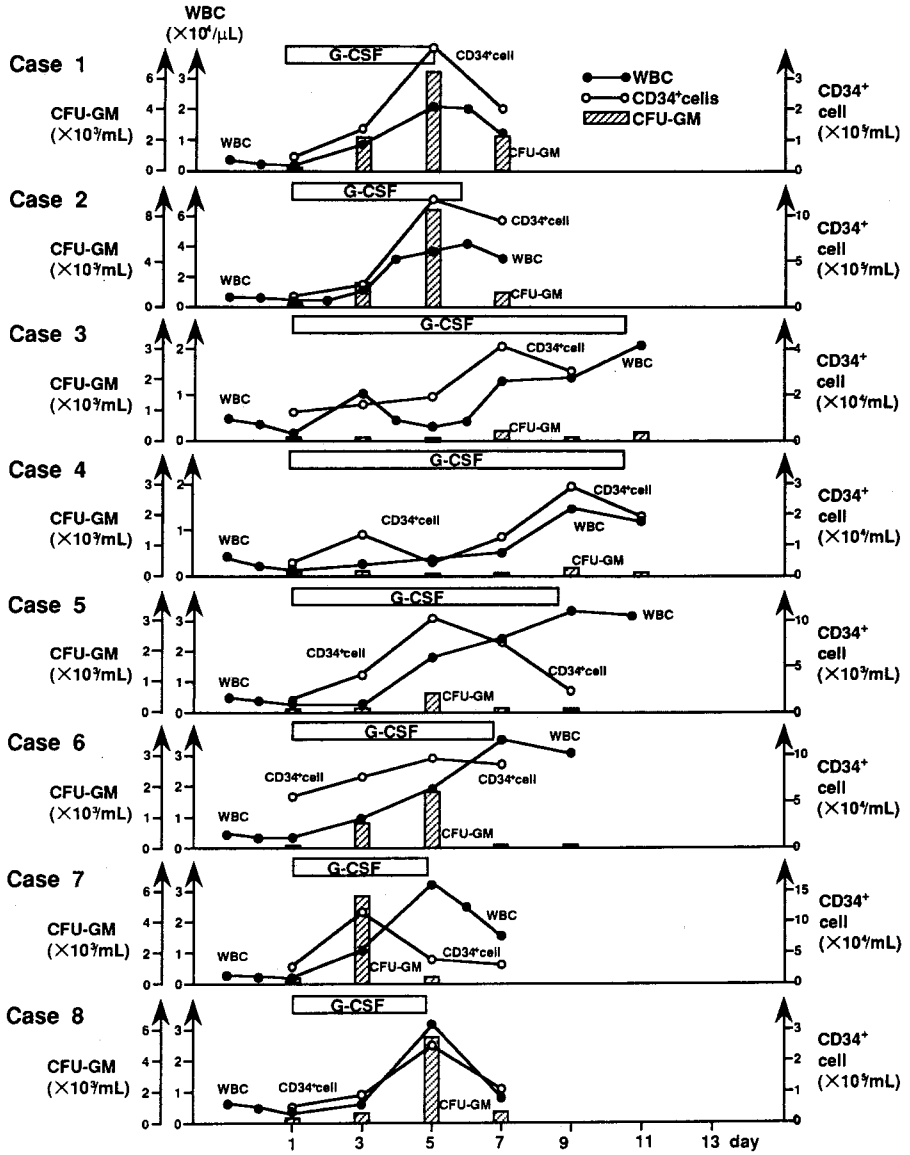

Fig. 2 Serial change of WBC count, $CD34^+$ cell count and CFU-GM count.

of CFU-GM and $CD34^+$ cell count ($P=0.0001$, $R=0.68$) but not with WBC.

Table 2 shows the summary on the recovery of CFU-GM in eight patients. Calculated recovery when leukapheresis volume is assumed to be 5L, was based on the efficiency of PBSC collection by Haemonetics V50 cell separator (13). If

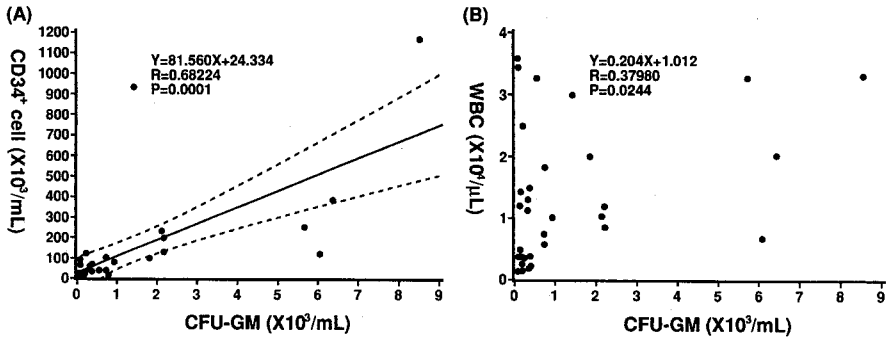

Fig. 3 Correlations between CFU-GM and $CD34^+$ cell (A) or WBC (B) in peripheral blood. The data are plotted relative to the analyses of 35 blood samples from eight patients in successive days.
There was a positive correlation between CFU-GM and $CD34^+$ cell ((A), $P=0.00001$).
Y equals the $CD34^+$ cell ($\times 10^3$/mL) (A), WBC ($\times 10^4/\mu$L) (B) and X equals the CFU-GM ($\times 10^3$/mL) count.

Table 2 *Recovery of CFU-GM*

Case	Maximum of CFU-GM(/mL)*	Maximum of CFU-GM($\times 10^5$/kg)**
1	6,200	4.6
2	8,100	5.4
3	144	0.16
4	148	0.16
5	550	0.4
6	1,848	1.7
7	5,636	5.0
8	5,208	4.6

*: actual recovery
**: calculated recovery when leukapheresis volume is assumed to be 5 L by a blood cell separator with 80% efficacy (13).

the leukapheresis is performed at the peak of CFU-GM, following equation may be applicable to calculate the obtainable colony numbers per Kg weight; [CFU-GM/Kg or BFU-E/Kg] = [CFU-GM or BFU-E/1$\times 10^5$ mononuclear cells] $\times$ [mononuclear cells/mL] $\times$ 5L (processed blood volume) $\times$ 0.8 (efficiency of PBSC collection by a blood separator (13)). Therefore, it is speculated that the recovery of CFU-GM was 1.7×10^5-5.4×10^5 colonies/Kg body weight by a blood cell separator, of which number is larger than needed for successful PBSCT (1.0×10^5 CFU-GM/Kg) (16, 17, 18, 23).

On the other hand, other three patients (case3-5) displayed poor recovery.

Table 3 *Influence of chemotherapy on recovery of CFU-GM*

case	Prior chemotherapy	Duration of prior chemotherapy(months)	Interval from last chemotherapy(months)	Chemotherapy at PBSC collection
1	CAP×1	1	1	CAP
2	PVeBV×1, CEB×2	3	1	CEB
3	cyclophosphamide* (etoposide+doxorubicin)×2, PBSCT(mitoxantoron+etoposide)	4	12	VMA
4	CAF×1	1	6	VMA
5	C-MOPP×6, PVP×3	9	1	ABVD
6	not done	0	0	CHOP
7	CHOP×1	1	1	CHOP
8	CHOP×1	1	1	CHOP

Abbreviations: CAP; cyclophosphamide, doxorubicin, cisplatin
PVeBV; cisplatin, etoposide, bleomycin, vinblastine CEB; carboplation, etoposide, bleomycin CAF; cyclophosphamide, doxorubicin, 5-FU C-MOPP; cyclophosphamide, vincristine, procarbazine, prednisone PVP; prednisone, etoposide CHOP; cyclophosphamide, doxorubicin, vincristine, prednisone VMA; vincristine, mytomycin-c, doxorubicin ABVD; doxorubicin, bleomycin, vincristine, dacarbazine
*: oral administration

In order to investigate the reasons for such insufficient recovery, we analyzed the influence of chemotherapy (Table 3). Five patients who obtained sufficient amount of PBSC had shorter duration of prior chemotherapy and shorter chemo-free period (interval from last chemotherapy) than those of other three patients. However, the three patients had poor recovery of PBSC by the same G-CSF administration procedure. Case 5 had longer prior chemotherapy period because 6 courses of C-MOPP therapy were conducted for complete remission, and had received salvage therapy after the relapse. Case 3 and 4 had VMA therapy, even though the prior chemotherapy duration was shorter and the chemo-free period was longer.

DISCUSSION

In order to collect sufficient amount of PBSC in a short period of time, we investigated the mobilization of stem cells from bone marrow to peripheral blood with 8 cancer patients, using G-CSF following myelosuppressive chemotherapy. In five pateints described here, the administration of G-CSF was followed by rapid increases in the numbers of WBC and PBSC. But in other three cases, the increase of WBC count was slow and the amplification of PBSC was insufficient.

The direct quantification of marrow-repopulating stem cells in humans is currently impossible. Therefore, at present, the CFU-GM count is employed as the most useful and reliable index to monitor hematopoietic reconstruction (2, 6,

8, 21). Accordingly, in this study, hematopoietic stem cells were quantified using a CFU-GM assay. We used a recombinant human IL3 and G-CSF for CFU-GM assay to minimize the interassay variation. The colony counts by the present method ranged 4 to $15/1\times10^5$ mononuclear cells at non-stimulated state and the number was compatible to that reported by McCarthy *et al* (9).

In general, the numbers of stem cells required for PBSCT is estimated to be $1.0\text{-}5.0\times10^5$ CFU-GM/kg (16, 17, 18, 23). In our institution, peripheral blood mononuclear cells containing 1.0×10^5 CFU-GM/Kg have been empirically verified to be sufficient for PBSCT (unpublished data). The present study showed that G-CSF administration amplified the stem cells in peripheral blood in five patients. Supposing 5L blood is processed by leukapheresis when the peak is attained, the recoveries of CFU-GM/Kg are calculated to be $1.7\times10^5\text{-}5.4\times10^5$. This indicates that only one leukapheresis (5L) may be sufficient to collect PBSC for successful PBSCT in these cases.

However, the assay of CFU-GM generally requires 14 days, and cannot be used to quantify the recovery of PBSC in a real time manner. Therefore, we tried to find some simpler parameter. A positive correlation was obtained between $CD34^+$ cell count and CFU-GM count (Table 2). It is known that $CD34^+$ cell appears in the human bone marrow only when marrow cells are undifferentiated (1, 4, 19, 25). Berenson *et al* (3) found that these cells manifest bone marrow reconstitutive capability when allogeneically transplanted into lethally irradiated baboons, suggesting that $CD34^+$ cell is developmentally close to pluripotent stem cell. In addition, $CD34^+$ cell count serves as a guide for estimating the quantity of CFU-GM available for collection from circulation (12). Our present study confirmed that the peak of $CD34^+$ cell coincided with that of CFU-GM (Fig. 2), and quantitatively significant positive correlation was noted between $CD34^+$ cell count and the CFU-GM count (Fig. 3). Therefore, the $CD34^+$ cell count can be used as an index for rapid monitoring of PBSC.

In 5 good recovery patients, the increase of $CD34^+$ cell occured 3-5 days after G-CSF administration. Therefore, days 3-5 after the commencement of G-CSF injection may be optimal timing for the cell harvesting.

On the other hand, 3 pateints did not respond well to use of G-CSF, in whom, the increase of WBC was slower and the maximum number of $CD34^+$ cell was lower than those of other five cases. Possible reasons include: (a) case 5 had longer duration of prior chemotherapy, (b) case 3 and 4 had recieved VMA therapy (the chemotherapy regimen including vincristine, doxorubicin and mitomycin-C) at PBSC collection time. Further evaluation is necessary to clarify this point.

Thus, in five of eight patients, a sufficient number of PBSC for transplanta-

tion can be collected in a short period by using G-CSF administration and the flow cytometry analysis peripheral $CD34^+$ cell can be used to quantify the recovery of PBSC in a real time manner.

REFERENCES

1. Andrew RG, Singer JW, Bernstein ID. Monoclonal antibody 12-8 recognized a 115-Kd molecule present on both unipotent and multipotent hematopoietic colony-forming cells and their precursors. Blood 1986, 67 : 842-845.
2. Bell AJ, Hamblin TJ, Oscier DG. Circulating stem cell autograft. Bone Marrow Transplant 1986, 1 : 103-110.
3. Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD, Bernstein ID. Antigen $CD34^+$ marrow cells engraft lethally irradiated baboons. J Clin Invest 1988, 88 : 951-955.
4. Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF, Shaper JH. Antigenic analysis of hematopoiesis III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-la cells. J Immunol 1984, 133 : 157-165.
5. Duhrsen U, Villeval JL, Boyd J, Kannourakis G, Morstyn G, Metacalf D. Effect of recombinant human granulocyte colony-stimulating factor on hematopoietic progenitor cell in cancer patients. Blood 1988, 72 : 2074-2081.
6. Juttner CA, To LB, Haylock DN, Dyson PG, Thorp D, Dart GW, Ho JQK, Horvath N, Bardy P. Autologous blood stem cell transplantation. Transplant Proc 1989, 21 : 2929-2931.
7. Kessinger A, Armitage JO, Landmark JD, Smith DM, Weisenburger DD. Autologous peripheral hematopoietic stem cell transplantation restores hematopoietic function following marrow ablative therapy. Blood 1988, 71 : 723-727.
8. Lasky LC. Hematopoietic reconstitution using progenitors recovered from blood. Transfusion 1989, 29 : 552-557.
9. McCarthy DM, Goldman JM. Transplantation of circulating stem cells. CRC Crit Rev Clin Lab Sci 1984, 20 : 1-24.
10. McNiece I, Andrews R, Stewart M, Clark S, Boone T, Quesenberry P. Action of Interleukin-3, G-CSF, and GM-CSF on highly enriched human hematopoietic progenitor cells : synergistic interaction of GM-CSF plus G-CSF. Blood 1989, 74 : 110-114.
11. Sheridan WP, Begley CG, Juttner CA et al. Effect of peripheral-blood progenitor cells mobilised by filgrastim (G-CSF) on platelet recovery after high-dose chemotherapy. Lancet 1992, 339 : 640-644.
12. Siena S, Bregni M, Brando B, Belli N, Ravagnani F, Gandola L, Stern AC, Lansdorp PM, Bonadonna G, Gianni AM. Flow cytometry for clinical estimation of criculating hematopoietic progenitors for autologous transplantation in cancer patiens. Blood 1991, 77 : 400-409.
13. Smith DM, Kessinger A, Landmark JD, Weisenburger DD, Armitage JO. Collection of peripheral blood stem cells using a modified elutriation protocol (abstr). Exp Haematol 1988, 16 : 486.
14. Stiff PJ, Murgo AJ, Wittes RE, Derist MF, Clarkson BD. Quantification of the peripheral

blood colony forming unit-culture rise following chemotherapy. Could leukocytaphereses replace bone marrow for autologous transplantation? Transfusion 1983, 23: 500-503.
15. Takaue Y, Watanabe T, Kawano Y, Koyama T, Abe T, Suzue T, Satoh J, Shimokawa T, Ninomiya T, Kosaka M, Shimizu E, Ogura T, Kuroda Y. Isolation and strage of peripheral blood hematopoietic stem cells for autotransplantation into children with cancer. Blood 1989, 74: 1245-1251.
16. Takaue Y. Peripheral blood stem cell autografts in children with lymphoma and acute lymphoblastic leukemia: Updated experience. Leuk Lymp 1991, 3: 241-256.
17. Tilly H, Vannier JP, Jean P, Bastit D, Monconduit M, Piguet H. Daily evaluation of circulating granulocyte-monocyte progenitors during bone marrow recovery from induction therapy in acute leukemia. Leuk Res 1986, 10: 353-356.
18. Tilly H, Bastit D, Luset JC, Esperou H, Monconduit M, Piguet H. Hematopoietic reconstitution after autologous peripheral blood stem cell transplantation in acute leukemia. Lancet 1986, 2: 154-155 (letter).
19. Tindle RW, Nichols RAB, Chan L, Campana D, Catovsky D, Birnie GD. A novel monoclonal antibody BI-3C5 recognizes myeloblasts and non-T non-B lymphoblasts in acute leukaemias and CGL blast crises, and reacts with immuture cells in normal bone marrow. Leuk Res 1985, 9: 1-9.
20. To LB, Haylock DN, Kimber RJ, Jutter CA. High levels of circulating haemopoietic stem cells in very early remission from acute non-lymphoblastic leukemia and their collection and cryopreservation. Br J Haematol 1984, 58: 399-410.
21. To LB, Juttner CA. Peripheral blood stem cell autografting: a new therapeutic option for AML? Br J Haematol 1987, 66: 285-288.
22. To LB, Juttner CA, Stomski F, Vadas MA, Kimber RJ. Immune reconstitution following peripheral blood stem cell autografting. Bone Marrow Transplant 1987, 2: 111 (letter).
23. To LB. Assaying the CFU-GM in blood: Correlation between cell dose and haematopoietic reconstitution. Bone Marrow Transplant 1990, 5: 16-18.
24. Richman CM, Weiner RS, Yankee RA. Increase in circulatng stem cells following chemotherapy in man. Blood 1976, 47: 1031-1039.
25. Watt SM, Karhi K, Gatter K. Distribution and epitope analysis of the cell membrane glycoprotein (HPCA-1) associated with human hematopoietic progenitor cells. Leukemia 1987, 1: 417-426.