

ORIGINAL ARTICLE

Differential role for very late antigen-5 in mobilization and homing of hematopoietic stem cells

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The role of very late antigen-5 (VLA-5) in homing and mobilization of hematopoietic stem cells from normal bone marrow (NBM) and bone marrow (MBM) and peripheral blood (MPB) from mobilized mice was investigated. We found a decreased number of VLA-5-expressing cells in the lineage-negative fraction of MPB. However, virtually all stem/progenitor cells were present in the VLA-5⁺ fraction and hence mobilization of hematopoietic stem cell subsets does not coincide with a downregulation of VLA-5. Stem/progenitor cells from MPB and MBM demonstrated enhanced stromal-derived factor-alpha-induced migration. This enhanced migration correlates with an improved hematopoietic reconstitution potential, with the migrated MPB cells showing the fastest reconstitution. Interestingly, homing of MPB, MBM and NBM stem/progenitor cells in bone marrow and spleen did not differ and is therefore not responsible for the differences in hematopoietic reconstitution. The observed increase in VLA-5⁺ cells in the recipients after transplantation can most probably be attributed to selective homing of VLA-5⁺ cells instead of an upregulation of VLA-5. Treatment with an antibody to VLA-5 partially inhibited bone marrow homing of progenitor cells, whereas homing in the spleen was hardly affected. These data indicate a differential role for VLA-5 in the movement of stem cells from and toward bone marrow.

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Introduction

In the treatment of cancer patients conditioning with high-dose chemotherapy followed by allogeneic or autologous

stem cell transplantation has been extensively exploited as a therapeutic modality. Although bone marrow is the main tissue for hematopoietic progenitor/stem cells, mobilized peripheral blood (MPB) stem cells have become the preferred source in the last decade.^{1–3} Collection of progenitor/stem cells after mobilization with chemotherapy or cytokines, or a combination of both has become a routine procedure in transplantation settings.^{4,5} The most important advantage of using MPB cells is the fact that a more rapid hematopoietic recovery is observed resulting in a shorter hospitalization and lower transfusion requirements.^{6–10}

The movement of hematopoietic stem cells from and toward the bone marrow is a multistep process, of which the underlying mechanisms are not completely understood. In the process of mobilization, hematopoietic stem cells will leave the stem cell niche in the bone marrow and migrate through the extracellular matrix. There is evidence that a number of adhesion molecules (AM) are involved in the migratory pathways during stem cell mobilization and homing. For instance, the major β 1-integrins, very late antigen (VLA)-4 and VLA-5 have been implicated in the adhesive interactions of stem cells with the bone marrow extracellular matrix and stromal cells by interacting with vascular adhesion molecule-1 (VCAM-1) and fibronectin present in the basal lamina.^{11–13} The role of VLA-4 in the processes of mobilization and homing has been extensively studied. It was found that the surface expression of this AM is downregulated in MPB cells compared to steady-state bone marrow hematopoietic cells.^{14–16} Moreover, anti-VLA-4 treatment augments progenitor and stem cell mobilization in mice.¹⁷ Mobilization is also induced with anti-VCAM-1 antibodies, suggesting a prominent role of VLA-4 in this process.¹⁸ In contrast, much less is known about the role of VLA-5 in the interaction of hematopoietic stem cells with the extracellular matrix. Although some data suggest a decreased expression of VLA-5 on MPB cells,^{16,19,20} others reported no differences in the level of expression on the surface of mobilized stem cell subsets.^{21,22}

Data from xenogeneic transplantation models suggest a crucial role for stromal-derived factor-alpha (SDF-1 α)²³ in the process of homing of stem cells. SDF-1 α is found to enhance adhesion of hematopoietic cells to endothelial cells and migration *in vitro*. Similar mechanisms may be involved in the process of homing. It is not unconceivable that for this inverse process of mobilization, the

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re-expression of the AMs on the transplanted cells is crucial. It was found that pretreatment of human CD34⁺ cells with antibodies that block VLA-4 partially reduced engraftment in non-obese diabetic/severe-combined immunodeficient (NOD/SCID) mice^{24,25} and fetal sheep.²⁶ Data on the role of VLA-5, however, are again rare and moreover contradictory. Although some reports mention a decrease in engraftment after VLA-5 blocking,^{24,27} no inhibitory effect on homing of progenitor cells is observed.^{17,28} It has also been reported that donor-derived chimerism is dependent on VLA-5 expression of the bone marrow graft and all long-term repopulating ability is confined to VLA-5-expressing cells.^{29,30} Furthermore, *in vitro* studies have tried to reveal the involvement of these AMs in the processes of mobilization and homing,^{31,32} although the correlation between levels of expression of VLA-5 and homing is not always clear.^{16,21} The clinical observations^{6–10} and our own experimental data³³ implicating that MPB cells engraft much faster than bone marrow stem cells are difficult to reconcile, assuming an opposite role for AMs in mobilization and homing.³⁴ If VLA-5 indeed plays an important role in the process of homing and the expression of this AM is downregulated during mobilization, either upregulation of this AM occurs within the first hours after transplantation of MPB cells or selective homing of cells expressing VLA-5 might be possible.

In this study, chemotherapy (cyclophosphamide) in combination with a cytokine (granulocyte-colony-stimulating factor (G-CSF)) was used to mobilize hematopoietic stem cell subsets as described before.³³ We compared the reconstitution potential of MPB cells with that of normal bone marrow (NBM) and bone marrow from mobilized animals (MBM) and analyzed the expression of various AMs in these cell populations. In particular, the involvement of VLA-5 in the opposing processes of mobilization and homing was determined. A difference in the *in vivo* distribution of the transplanted cells might be responsible for differences in the initiation of marrow reconstitution. Hence, we studied the homing efficiency of grafts consisting of either NBM, MBM or MPB stem cells to bone marrow and spleen of irradiated recipients in the first 24 h after transplantation. Besides the involvement of AMs, it is also reported that the expression of the CXCR4 receptor on hematopoietic stem cell subsets is a determinant factor in the process of homing to the bone marrow.^{35,36} Therefore, we also determined the SDF-1 α -induced migration of the NBM, MBM and MPB cells and subsequently the engraftment rate of the cells in the migrated fraction.

Materials and methods

Mice

In this study, female CBA/H mice were used as source of hematopoietic progenitors and stem cells. The mice were bred at the Netherlands Energy Research Foundation, Petten, The Netherlands and maintained under clean conventional conditions in the animal facilities of the University of Groningen, The Netherlands. The mice were fed *ad libitum* with food pellets and acidified tap water

(pH = 2.8). Unless otherwise stated, mice were splenectomized under halothane/O₂-anesthesia and allowed to recover for at least 2 weeks.

Hematopoietic cells

Bone marrow cells were obtained by flushing the femoral cell content with Iscove's modified Dulbecco's minimum essential medium (IMDM, GibcoBRL, Paisley, Scotland) supplemented with 5% fetal calf serum (FCS, GibcoBRL, Paisley, Scotland). The cell suspensions were dispersed through a 21-gauge needle. Peripheral blood cells were harvested from the retro-orbital venous plexus after a single intraperitoneal injection of cyclophosphamide (Aldrich-Chemie, Steinheim, Germany) at a dose of 200 mg/kg at day 0 and after subcutaneous injections of 2 \times 25 μ g/mouse of PEG-rHu-G-CSF (Amgen Inc., Thousand Oaks, CA, USA), given at days 3 and 6 after cyclophosphamide injection, followed by harvesting the peripheral blood cells at day 9. Approximately 1 ml blood was collected into 4 ml IMDM supplemented with 5% FCS and heparin (25 IU). The collected blood cell suspension (5 ml) was centrifuged over an equal volume of Lympholyte-M (Cedarlane Laboratories Ltd, Hornby, Canada) at 600 g (1650 r.p.m. in R2000B rotor) for 30 min at room temperature. After centrifugation, the mononuclear cells within the opaque interface layer were isolated and washed in IMDM/5% FCS for 10 min at 400 g. Nucleated cell counting was performed on a Coulter Counter Model Z22 (Coulter Electronics, Hialeah, FL, USA). The cell suspensions were kept on ice until use.

In vitro migration assay

This assay was performed in 5- μ m pore transwell systems (Corning Inc., Corning, NY, USA). Bone marrow or peripheral blood cells (5 \times 10⁶/ml) were loaded into the upper well of the transwell system. A total volume of 100 μ l was applied to the upper well. The lower well was filled with 600 μ l IMDM medium supplemented with 100 ng/ml rmSDF-1 α (R&D Systems, Minneapolis, MN, USA). The transwell systems were incubated for 4 h at 33°C in a 5% CO₂ humidified atmosphere. Next, the upper wells were carefully removed and the cells in both compartments were collected. The cells were washed and resuspended in IMDM/5% FCS. The number of hematopoietic progenitor and stem cells were assayed by culturing cells from the migrating and non-migrating fractions in the colony-forming cell assays as described below.

Colony-forming unit assays

Progenitor cells were assayed as described earlier.³³ Briefly, cells were plated out in alpha-medium (StemCell Technologies Inc., Vancouver, BC, Canada) containing 0.8% methylcellulose (Fluka, Bachs SG, Switzerland), 30% FCS (GibcoBRL, Paisley, Scotland) and 10⁻⁴ mol/l 2-mercapthoethanol (Merck, Darmstadt, Germany) at concentrations varying from 10⁴ to 5 \times 10⁵ nucleated cells/ml. Colony growth was stimulated by granulocyte-macrophage-CSF and stem cell factor. Cultures were plated in 35-mm polystyrene culture dishes (Falcon, Becton-Dickinson, Lincoln Park, NJ, USA) and grown at 37°C in a 5%

CO₂ humidified atmosphere. Colonies (>50 cells) were scored after 7 days of culture.

The cobblestone area-forming cell (CAFC) assay allows the assessment of primitive hematopoietic stem cells. The CAFC assay was performed by establishing confluent FBMD-1 cell cultures in 96-well plates (Corning Inc., Corning, NY, USA). The confluent cell cultures were overlaid with mobilized peripheral blood cells in a limiting dilution set up. Eight twofold dilutions were used with 10 replicate wells per dilution. The cells were cultured in IMDM supplemented with 20% horse serum (GibcoBRL, Paisley, Scotland) at 33°C in a 10% CO₂ humidified atmosphere with a half-volume medium change every week. The percentage of wells with at least one phase-dark hematopoietic clone of at least five cells beneath the stromal layer was determined 4–5 weeks after initiating the culture. Cobblestone area frequencies were calculated using Poisson statistics.³⁷

Hematopoietic reconstitution assays

The hematopoietic reconstitution rate was determined after transplanting different cell doses of bone marrow and peripheral blood cell suspensions in groups of five lethally irradiated CBA/H mice. Mice were irradiated with 10.0 Gy γ -rays (0.89 Gy/min) in a ¹³⁷Cs source (IBL 639, CIS Bio International, Gif-sur-Yvette, France), 24 h before transplantation. Mice were bled (40 μ l) from the retro-orbital sinus on weekly basis and blood cell counts were performed on a Coulter Counter Model Z2. For the red and white blood cells, a 100 μ m aperture tube was used while the platelet counts were performed with a 70 μ m aperture tube.

In vivo homing assay

The homing efficiency of hematopoietic progenitor and stem cells was assayed by transplanting cell suspensions into lethally irradiated mice as described above. Three and 24 h post transplant bone marrow and spleen cells of the recipients were isolated and plated out in the colony-forming assays. In measuring homing to the bone marrow, cells from one femur were assumed to represent about 7% of the entire organ.³⁸ The effect of VLA-5 blocking on homing to the bone marrow and spleen was assayed by incubating the cell suspension with purified rat anti-mouse CD49e monoclonal antibody (30 min at room temperature). After washing, the cells were transplanted into lethally irradiated recipients as described for the hematopoietic reconstitution assay. At 3 h post transplant, bone marrow and spleen cells of the recipients were isolated and plated out in the colony-forming assays.

Flow cytometric cell sorting and analysis of AM expression on Lin⁻ cells

For the detection of AM expression bone marrow or mononuclear peripheral blood cells were stained with biotinylated antibody CD3e, CD11b, CD45R/B220, Ly-6G and TER-119 to allow the selection of lineage-negative (Lin⁻) cells. Antibodies were visualized by streptavidin-Alexa Fluor. Cells were furthermore stained with r-phycoerythrin (PE)-conjugated antibodies recognizing one of the following: CD11a (leukocyte-function-

associated antigen 1 (LFA-1), clone 2D7), CD43 (Leukosialin, clone S7), CD44 (homing-associated cell AM (HCAM), clone IM7), CD49d (VLA-4, clone 9C10), CD49e (VLA-5, clone 5H10-27; MFR5) or CD62L (L-selectin, clone MEL-14). The detection of the VLA-5-expressing cells after transplantation was assayed by labeling the graft with the green fluorescent dye PKH67-GL (Sigma, Steinheim, Germany). At 3 and 24 h post transplant, bone marrow and spleen cells of the recipient mice were isolated and stained with the biotinylated lineage-specific antibodies as described above. In this case, the antibodies were developed with streptavidin-PerCP. Then, the cells were stained with r-PE-conjugated CD49e (VLA-5, clone 5H10-27). All antibodies were obtained from BD Biosciences/PharMingen (Heidelberg, Germany). Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Erembodegem, Germany) to yield the percentage of AM⁺ Lin⁻ cells. Separation of VLA-5⁺ and VLA-5⁻ subfractions was performed after staining with the PE-conjugated CD49e antibody described above and using a MoFlow flow cytometer (Cytomation Inc., Fort Collins, CO, USA).

Statistical analysis

Data are expressed as the mean \pm s.e.m. Differences between groups were analyzed by means of an unpaired two-sided *t*-test. A *P*-value of less than 0.05 was considered significant.

Results

Expression of AMs on bone marrow and peripheral blood cells

First, the effect of mobilization on the expression of AMs was investigated. In Figure 1, the percentage of positive cells for a series of AMs in the Lin⁻ fraction of mononuclear cells from MPB, MBM and NBM is depicted. No significant differences in the percentage of CD43/

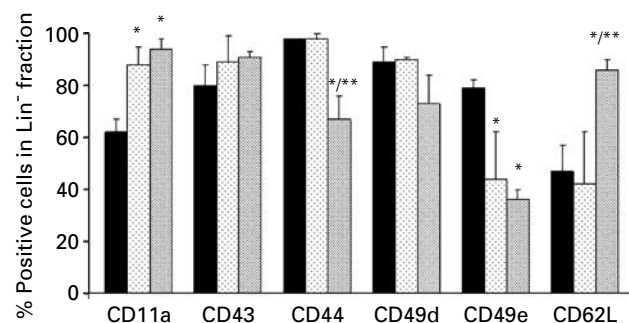


Figure 1 Expression of AMs on bone marrow and peripheral blood cells after mobilization. The expression of six AMs on Lin⁻ cells was determined by flow cytometry as described in 'Materials and methods'. CD11A = LFA-1, leukocyte-function-associated antigen, CD43 = leukosialin, CD44 = HCAM, homing-associated cell adhesion molecule, CD49d = VLA-4, very late antigen-4, CD49e = VLA-5, very late antigen-5, CD62L = L-selectin. Data represent the mean percentage of positive cells \pm s.e.m. *n* = 3. *Significantly different (*P* < 0.05) from NBM. **Significantly different (*P* < 0.05) from MBM. ■, NBM; □, MBM; ▒, MPB.

leukosialin- and CD49d/VLA-4-expressing cells could be observed in all three sources. The percentage of CD11a/LFA-1-expressing cells was increased in MBM and MPB. In comparing the expression of the various AMs between MBM and MPB cells, a significant decrease for CD44/HCAM and an increase for CD62L/L-selectin-expressing cells in MPB was noticed. The percentage of CD49e/VLA-5-expressing cells in the Lin⁻ fraction of both MBM and MPB was significantly decreased. Downregulation of AM expression would be in line with the general hypothesis that decreased levels of AM expression are relevant to the process of mobilization. The observed strong decrease in the number of VLA-5⁺ cells after mobilization combined with the limited knowledge on the role of VLA-5 in hematopoietic stem cell mobilization and homing urged us to focus on the role of this integrin.

Reconstitution ability of mobilized stem and progenitor cells

Next, we compared the *in vivo* reconstitution kinetics of MPB with that of cells from NBM and MBM. In Figure 2, reconstitution curves for white blood cells are shown. The deeper nadir after transplanting MBM cells might be explained by the lower number of progenitor cells in the graft compared with NBM. The number of progenitor cells in the MPB graft, however, is somewhat lower than in the NBM graft, but still the reconstitution rate is higher as demonstrated by a less deep nadir and a faster recovery to normal levels of white blood cells. For red blood cells and platelets comparable differences were noticed (data not shown). The observed differences in engraftment rate might be caused by differences in the ability of cells to migrate across the vascular endothelial layer into the extravascular hematopoietic tissue. We therefore analyzed the migration capacity of the progenitor and stem cells from NBM, MBM and MPB. In Table 1, the results of the SDF-1 α -induced

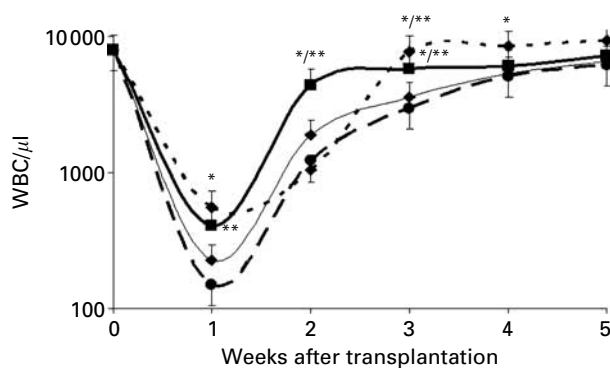


Figure 2 Reconstitution curves for white blood cells after transplantation. The hematopoietic engraftment rate was determined in groups of five lethally irradiated CBA/H mice transplanted with 2×10^5 NBM, MBM, MPB or MPB-M cells. Mice were irradiated 24 h before transplantation. Recipients were bled from the retro-orbital sinus on weekly basis and blood cell counts were performed. Shown are the mean \pm s.e.m. number of white blood cell counts. *Significantly different ($P < 0.05$) from NBM. **Significantly different ($P < 0.05$) from MBM. (Progenitor cells) —◆—: NBM, ± 700 ; ---■---: MBM, ± 330 ; —■—: MPB, ± 550 ; ---◆---: MPB-M, ± 60 . (Stem cells) —◆—: NBM, ± 40 ; ---■---: MBM, ± 40 ; —■—: MPB, ± 90 ; ---◆---: MPB-M, ± 15 .

migration are shown. In the 4-h time period, $< 1\%$ of the progenitor cells from NBM migrate, whereas in MBM the migration capacity increased to around 7%. In MPB, the migration of progenitor cells further increased to 20%. In MBM, 23% of the CAFC-d35 migrated, whereas in MPB, an increase to about 34% in the migration of stem cells could be observed. When migrated cells from MPB (MPB-M) were transplanted, a graft consisting of almost six- to 10-fold less stem and progenitor cells as compared with the MPB graft resulted in a comparable reconstitution rate (Figure 2). It should be noted here that the number of VLA-5-expressing cells in the MPB-M fraction further decreased to $< 5\%$ (data not shown).

VLA-5 expression on hematopoietic progenitor/stem cells

Because of the apparent inverse correlation between VLA-5 expression on Lin⁻ cells and their hematopoietic reconstitution potential, it is relevant to determine the VLA-5 expression on hematopoietic progenitors and stem cells. To this end, MPB cells were sorted into VLA-5⁺ and VLA-5⁻ fractions immediately after harvesting the mononuclear cell fraction from peripheral blood and plated out in colony-forming unit-GM and CAFC cultures. It is clear from Table 2 that virtually all hematopoietic stem cell subsets

Table 1 Migratory capacity to an SDF-1 α gradient of NBM and MBM and MPB after mobilization

	% Migration	
	CFU-GM	CAFC-d35
NBM	0.9 \pm 0.6	ND
MBM	6.8 \pm 1.6*	23.0 \pm 3.0*
MPB	19.8 \pm 2.9**/**	33.5 \pm 4.5**/**

Abbreviations: CAFC = cobblestone area-forming cell; CFU-GM = colony-forming unit-granulocyte-macrophage; MBM = bone marrow from mobilized animal; MPB = mobilized peripheral blood; ND = not detectable; NBM = normal bone marrow; SDF-1 α = stromal-derived factor- α .

SDF-1 α (100 ng/ml) was added in the lower chambers of transwell plates, with mobilized cells (5×10^6 /ml) in the upper chamber. After 4 h at 37°C, the upper chambers were removed and the migrated and non-migrated cells were plated out in the colony-forming assays. The percentage migration was calculated as number of colony-forming cells in migrating fraction divided by total number of colony-forming cells (in both migrating and non-migrating fractions). Data are corrected for spontaneously migrating cells. Results represent the average \pm s.e.m. of three experiments.

*Significantly different ($P < 0.05$) from NBM.

**Significantly different ($P < 0.05$) from MBM.

Table 2 Distribution of progenitor cells and stem cells between VLA-5⁺ and VLA-5⁻ fraction of MPB cells

	CFU-GM (%)	CAFC-d35 (%)
VLA-5 ⁺ fraction	98.8 \pm 0.5	99.4 \pm 0.7
VLA-5 ⁻ fraction	1.3 \pm 0.5	0.7 \pm 0.7

Abbreviations: CAFC = cobblestone area-forming cell; CFU-GM = colony-forming unit-granulocyte-macrophage; VLA = very late antigen. MPB cells were harvested and immediately after collection stained with PE-conjugated anti-mouse CD49. Cells were sorted in VLA-5⁺ (R2) and VLA-5⁻ (R4) fractions and plated out in the colony-forming assays as described in 'Materials and methods'. Data are mean \pm s.e.m. $n = 2$.

were detected in the VLA-5⁺ fraction. Hence, it can be concluded that mobilization of hematopoietic progenitor/stem cells does not coincide with downregulation of VLA-5 in cells with progenitor or stem cell activity.

Homing efficiency of MPB cells and bone marrow stem cells

The increased migration to SDF-1 α (Table 1) of progenitor/stem cells from MPB cells might facilitate their homing and thereby enhance the reconstitution potential. Therefore, we compared the homing capacity of mobilized progenitor/stem cells with those from bone marrow. NBM, MBM and MPB cells were transplanted into lethally irradiated syngeneic recipients. Three and 24 h later, bone marrow and spleen cells of the recipients were isolated and plated out in the clonogenic assays. Figure 3 shows the homing efficiencies of progenitor/stem cells in both organs. In bone marrow, no significant differences in homing efficiency could be detected for progenitor cells, irrespective of their source and time point after transplantation (Figure 3a). For the stem cells, again no significant differences could be detected, although their overall homing efficiency was higher than that of progenitor cells. In the spleen, the homing efficiencies of the progenitor cells at 24 h post transplant were somewhat lower compared with the 3-h time point, but again no significant differences between the

sources could be demonstrated (Figure 3b). Stem cells from NBM, however, had a significantly lower homing efficiency in the spleen than those from MBM and MPB. As the reconstitution potential of migrated cells from MPB exceeded that of unfractionated MPB cells, we also determined the homing efficiency of stem cells in the SDF-1 α -induced migrated cell fraction. A twofold increase in homing of MPB-M cells was observed in the bone marrow (data not shown). In the spleen of these recipients, no such difference was seen. We were unable to assess whether this increase also represents an increase in progenitor/stem cell homing because of the low cell yield. Based on these data, however, the observed differences in reconstitution potential between MPB and NBM or MBM cells cannot be explained by differences in homing efficiencies.

Role of VLA-5 expression during homing of hematopoietic stem cells

In order to study the role of VLA-5 during the process of homing, MPB cells were labeled with the green fluorescent dye PKH67-GL before transplantation and VLA-5 expression was measured on the donor-derived (PKH⁺) cells that engrafted in bone marrow or spleen. Figure 4 shows the percentage of VLA-5-expressing Lin⁻ cells in bone marrow, spleen and peripheral blood of the recipient during the first 24 h after transplantation of MPB cells. In bone marrow and spleen, an increase in the percentage of VLA-5-expressing cells could be noticed within the first 3 h after transplantation, after which the levels of VLA-5⁺ cells leveled off. For MPB-M cells comparable kinetics could be observed (data not shown). The next question we addressed was whether this fast increase, which was particularly prominent in the bone marrow, is caused by an upregulation of VLA-5 or by selective or preferential homing of VLA-5-expressing cells. To this end, we measured the

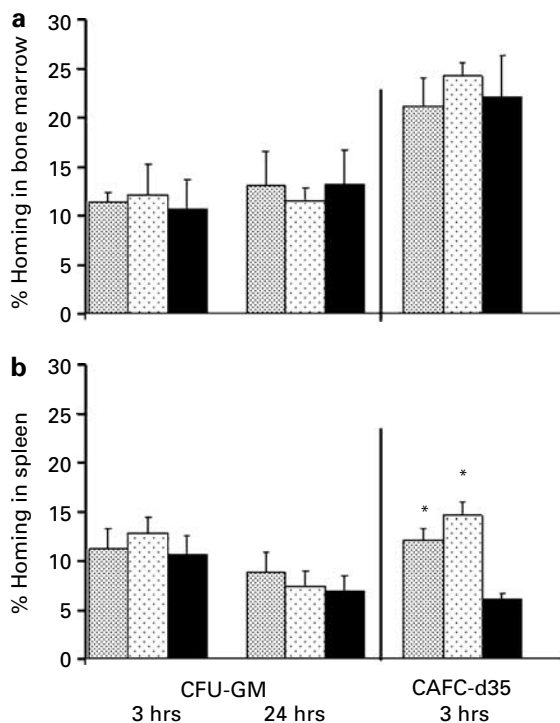


Figure 3 Homing of hematopoietic progenitor cells and stem cells in bone marrow and spleen. Lethally irradiated mice were transplanted with 10^7 – 10^8 NBM (■), MBM (▨) or MPB (□) cells. Three or 24 h later, bone marrow and spleen cells from the recipient were isolated and the recovery of donor-derived progenitor cells and stem cells were quantified by colony-forming assays. Shown are the mean \pm s.e.m. percentage of homing in bone marrow (a) and spleen (b). $n = 3$ for stem cells and $n = 6$ for progenitor cells. *Significantly different ($P < 0.05$) from NBM.

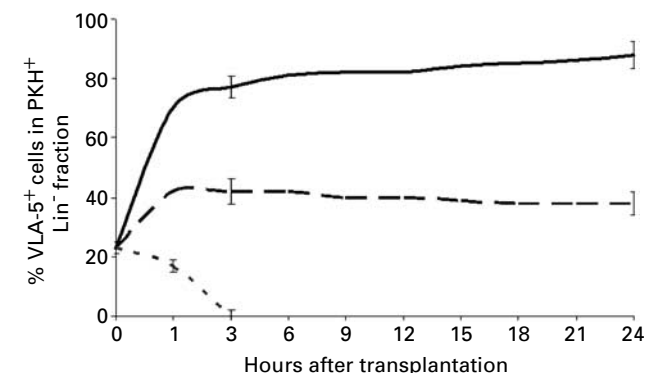


Figure 4 Percentage of donor-derived VLA-5⁺ cells after transplantation. PKH67-GL-labeled cells were transplanted in lethally irradiated animals. Three and 24 h post transplant bone marrow, spleen and peripheral blood cells were isolated and stained with biotinylated antibodies CD3e, CD11b, CD45R/B220, Ly-6G and TER-199 (streptavidin-PerCP), followed by staining with PE-conjugated CD49e. Cells were analyzed by flow cytometry and the percentage of donor-derived PKH⁺ Lin⁻ VLA-5⁺ cells determined. Lines represent mean \pm s.e.m. of positive cells in bone marrow (—), spleen (---) or peripheral blood (· · · · ·) of lethally irradiated mice. $n = 6$.

number of VLA-5-expressing cells in the peripheral blood of the recipients immediately after transplantation. As shown in Figure 4, the percentage of VLA-5⁺ cells in peripheral blood rapidly decreased to undetectable levels within 3 h. These data do not support the notion of upregulation of VLA-5 shortly after transplantation. Moreover, in Figure 5, a comparison between the calculated (i.e. expected) and observed levels of donor-derived VLA-5⁺ cells is shown after transplanting MPB or MPB-M cells. The calculated levels are based on the assumption that only VLA-5⁺ cells will home to the different organs (see legend Figure 5). The detected levels closely resemble those of the calculated ones. All these data are in favor of a selective homing of VLA-5-expressing cells after transplantation.

Effect of blocking VLA-5 receptor on hematopoietic reconstitution and homing of stem cells

Finally, experiments were conducted to study the effect of blocking VLA-5 expression on cell homing. Next to MPB cells, also MBM cells were included in these experiments. After VLA-5 blocking of MPB cells, an inhibition of 53 ± 6% in the homing of progenitor cells in bone marrow could be found, whereas homing of these subsets in the spleen of the recipients was only inhibited by 14 ± 4% (Table 3). For MBM cells, the inhibition of homing in bone marrow was comparable with that of MPB cells, whereas homing in the spleen was not affected at all after VLA-5 blocking. The impact of blocking VLA-5 expression on

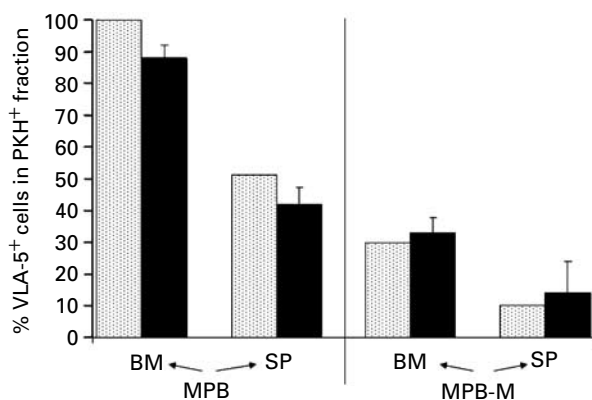


Figure 5 Comparison between calculated and observed homing of PKH⁺ Lin⁻ VLA-5⁺ cells. MPB and MPB-M cells were labeled with PKH67-GL and transplanted in lethally irradiated animals. At 3 h post transplant, bone marrow and spleen cells were isolated and stained with biotinylated antibodies CD3e, CD11b, CD45R/B220, Ly-6G and TER-199 (streptavidin-PerCP), followed by staining with PE-conjugated CD49e. Cells were analyzed by flow cytometry and the percentage of donor-derived PKH⁺ Lin⁻ VLA-5⁺ cells determined (■). Calculated levels of homing (□) were based on the assumption that only VLA-5⁺ cells will home to the hematopoietic organs. In these experiments, the initial percentage of VLA-5⁺ cells in the graft was 14 ± 3% for MPB and 3.0 ± 0.6% for MPB-M. The homing efficiencies for Lin⁻ PKH⁺ cells was 10 ± 1% in the bone marrow and 29 ± 4% in the spleen. So, if 10% of MPB cells home in BM of the recipient and 14% of the graft is VLA-5⁺ all of the grafted cells will be VLA-5⁺. In the spleen of the recipient, 29% of the transplanted cells will home. If only VLA-5⁺ cells will home this percentage will be around (14/29) × 100 = 48%. For MPB-M, the calculated levels are (3/10) × 100 = 30% and (3/29) × 100 = 10%, respectively. Data are mean ± s.e.m. n = 3.

engraftment was explored by determining the reconstitution rate after treating MPB cells with an antibody to VLA-5 and subsequently transplanted in lethally irradiated animals. Blocking VLA-5 expression resulted in a deeper nadir at 1 week after transplantation and a delay in white blood cell reconstitution (Figure 6). For red blood cells and platelets comparable effects were noticed (data not shown).

Discussion

Initially, both autologous and allogeneic transplantations were performed using bone marrow cells. During the last decade, however, MPB cells collected by apheresis^{39,40} have largely replaced NBM as source of hematopoietic stem cells. Although cytokines such as G-CSF are commonly used in allogeneic donors, patients undergoing autologous transplantation are mobilized following chemotherapy. In

Table 3 Effect of VLA-5 blocking on homing of progenitor cells in bone marrow and spleen

	% Inhibition at 3 h post transplantation	
	In bone marrow	In spleen
MPB	53 ± 6	14 ± 4
MBM	60 ± 12	0

Abbreviations: MBM = bone marrow from mobilized animal; MPB = mobilized peripheral blood; VLA = very late antigen.

MPB and MBM cells were incubated with or without an antibody to VLA-5 and subsequently transplanted in lethally irradiated mice (10⁵ nucleated cells/mouse). Mice were lethally irradiated 24 h before transplantation. Three hours post transplant, the bone marrow and spleen cells from the recipients were isolated and plated out in colony-forming assay (CFU-GM). Data shown are mean ± s.e.m. n = 3.

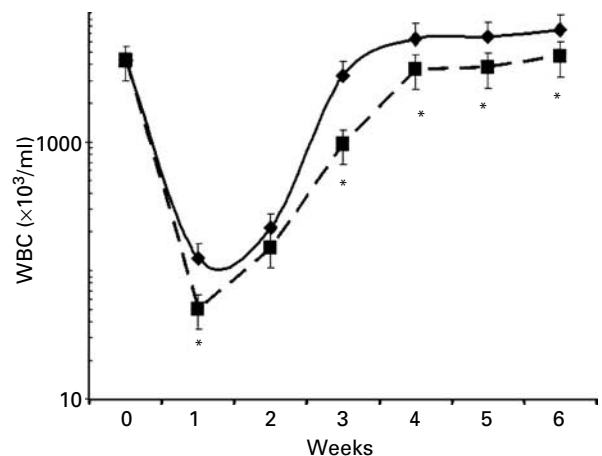


Figure 6 Impact of VLA-5 blocking on engraftment of MPB cells. MPB cells were incubated with or without an antibody to VLA-5 and subsequently transplanted in a group of five lethally irradiated mice (10⁵ nucleated cells/mouse). Recipient mice were lethally irradiated 24 h before transplantation. Recipients were bled from the retro-orbital sinus on weekly basis and blood cell counts were performed. Retrospectively, it was calculated that the untreated graft consist of 147 progenitor cells and the treated graft of 137 progenitor cells. Data shown are mean ± s.e.m. n = 2. *Significantly different (P < 0.05) from NBM.

this study, a combination of cyclophosphamide and G-CSF was used to mobilize stem and progenitor cells in splenectomized animals. Hematopoietic stem cell subsets express specific adhesion or homing receptors that ultimately determine hematopoietic regeneration after stem cell transplantation. We therefore investigated the role of the AM VLA-5 in homing and engraftment of mobilized stem cells. First, the effect of this mobilization regimen on the expression of a series of AMs was determined on the Lin⁻ cells from MBM and MPB cells (Figure 1). Although we were hampered by the fact that the mouse strain used in this study does not allow the selection for Sca-1-expressing cells,⁴¹ we could confirm the data of Orschell-Traycoff *et al.*²⁹ with respect to the expression of AMs on Lin⁻ Sca-1⁺ cells from NBM cells. The observed decrease in the percentage of VLA-5-expressing cells after mobilization was also found by others¹⁴ and might suggest a downregulation of this integrin. This downregulation, however, does not occur on the hematopoietic stem cell subsets as virtually all stem and progenitor cells could be detected in the VLA-5⁺ fraction (Table 2). Hence, VLA-5 downregulation is not a prerequisite for mobilization of hematopoietic stem cells. These data are in accordance with those reported earlier.^{29,42}

It has been reported that MPB cells demonstrate an accelerated hematopoietic recovery after transplantation.^{6–10} Figure 2 indeed shows an enhanced hematopoietic recovery after transplantation with MPB cells, and in particular the SDF1 α -induced migrated cells, in comparison with bone marrow cells from control and mobilized mice. The repopulation curves of the MPB and MPB-M cells are characterized by a less deep nadir. Apparently, the initial phase after transplantation, most obviously the process of homing and engraftment, determines this difference in hematopoietic repopulation. As it is thought that engraftment is initially mediated by progenitor cells, these cells might be responsible for the observed differences. We have to recognize, however, that some contradictory data exist on the role of the progenitor cells in the early phase of engraftment.^{43–45} Although in a bone marrow graft from normal or mobilized mice the ratio of progenitor/stem cell changed from ± 16 to ± 8 , respectively, transplanting a comparable number of stem cells showed retarded hematopoietic repopulation using MBM compared to NBM (Figure 2). Owing to the change in the ratio of the stem cell subsets, in the MBM graft only half the number of progenitor cells is present. That might be the explanation for the observed effect and is supportive for a prominent role of the committed progenitors in the early phase of hematopoietic reconstitution. It has been shown that migrated cells gave significantly higher levels of engraftment compared with non-migrating cells.^{23,35} Our data confirm these observations by demonstrating a striking difference in repopulation capacity between MPB and MPB-M grafts. Although in the MPB-M graft the number of both progenitors and stem cells is much lower compared with MPB cells, its reconstitution rate is more or less comparable. As stated before, the differences in reconstitution rate cannot be contributed to differences in VLA-5 expression because all progenitors and stem cells are present in the VLA-5⁺ fraction (Table 2).

An alternative explanation for the observed differences in the rate of reconstitution following MPB or NBM/MBM cell transplantation would be the difference in lodging of stem cell subsets to the hematopoietic organs of the recipient. We were, however, unable to detect any difference in homing efficiency between the different stem cell sources in both bone marrow and spleen (Figure 3a and b). Homing of progenitor cells in bone marrow plateaued after 3 h. Although in the spleen initially a comparable homing was observed, it declined thereafter. These findings are in agreement with those of Szilvassy *et al.*,⁴⁶ although these authors did observe a difference in homing efficiency between NBM and MPB cells.¹⁶ For primitive hematopoietic stem cells similar homing kinetics were found from different sources. It should be noted, however, that stem cells homed more efficiently to the bone marrow of the recipient in accordance with previous reports.^{47,48}

It could be shown that during the first 3 h post transplant, an increase in VLA-5⁺ cells was found in both the bone marrow and spleen of the lethally irradiated recipients (Figure 4). Although this fast increase suggests an upregulation of VLA-5, we have strong evidence that rather this increase is caused by selective homing of VLA-5-expressing cells. Firstly, we were unable to demonstrate an increase in VLA-5⁺ cells in the peripheral blood of the recipients during this early period (Figure 4). Secondly, calculations based on the homing of donor-derived cells and the initial number of VLA-5-expressing cells in the graft revealed expected levels of VLA-5-expressing cells, assuming a selective homing of VLA-5⁺ cells from unfractionated or migrated cells from MPB cells (Figure 5). Taken together, these findings make it very unlikely that any upregulation of VLA-5 occurs and that the observed increase in VLA-5-expressing cells after transplantation is caused by preferential/selective homing of VLA-5-expressing cells.

Further evidence for a prominent role of VLA-5 in the process of homing and engraftment in the bone marrow is demonstrated by the inhibiting effects of an antibody to VLA-5 (Table 3, Figure 6). A striking observation was the absence of an inhibitory effect of VLA-5 blocking on the homing in the spleen. In some reports, the inhibitory effect of VLA-5 blocking was determined either by measuring the number of CD34⁺ cells from human cord blood homed in the bone marrow or spleen,²⁴ or by the quantifying the engraftment in bone marrow of NOD/SCID mice expressed as % CD45 cells.^{23,24} Others measured the effect of VLA-5 blocking more directly by determining the homing of hematopoietic progenitor cells in bone marrow. Craddock *et al.*¹⁷ did not observe any alteration in bone marrow lodging of progenitor cells at 3 h post transplant, whereas others detected inhibiting levels comparable to ours.²⁷ So far, however, we are unaware of any study in which the homing efficiency of progenitor cells in the spleen was investigated. In this study, engraftment was ascertained by determining hematopoietic reconstitution after transplantation. Blocking VLA-5 expression on MPB cells resulted in a delay in white blood cell reconstitution in the recipients (Figure 6). As we could demonstrate that blocking of VLA-5 expression did not inhibit homing in the spleen (Table 3), these effects can only be attributed to the inhibiting effect

on bone marrow homing. Moreover, these data indicate that hematopoietic stem cells that homed to the bone marrow are responsible for the short-term hematopoietic reconstitution, which is in line with the finding that hematopoietic stem cell subsets that home to the spleen mainly contribute to long-term hematopoiesis.⁴⁶

In summary, our study shows that mobilization of hematopoietic stem cells with cyclophosphamide and G-CSF does not cause a downregulation of VLA-5. The MPB cells, and especially those cells migrating to SDF-1 α , demonstrate an enhanced reconstitution potential. A rapid increase in donor derived VLA-5-expressing cells after MPB transplantation is seen, which is caused by selective homing of VLA-5⁺ cells. The crucial role of VLA-5 in the process of homing was demonstrated by the observation that blocking VLA-5 significantly decreased bone marrow homing while homing to the spleen is not affected. These data are indicative for different adhesive pathways in the process of homing to bone marrow and spleen. To elucidate the underlying mechanism for the differences in reconstitution potential further investigations are needed.

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