Modulation of homing properties of primitive progenitor cells generated by *ex vivo* expansion

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Background and Objectives. The maintenance of adequate interactions with the bone marrow (BM) microenvironment is critical to ensure efficient homing of *ex vivo*-expanded hematopoietic cells. This study was intended to assess adhesion and migration properties of long-term culture-initiating cells (LTC-IC) harvested after self-renewal division in *ex vivo* culture and to determine their susceptibility to growth-inhibitory signals mediated by adhesion to BM stromal ligands.

Design and Methods. We used cell tracking to isolate primitive LTC-IC that had accomplished 1 or 2 divisions *ex vivo*. Adhesion, migration and growth inhibition of divided LTC-IC were determined in the presence of purified BM ligands, and compared to the properties of uncultured LTC-IC.

Results. As compared to undivided LTC-IC, adhesion and migration mediated by very late antigen (VLA)-4 integrin across both vascular cell adhesion molecule-1 (VCAM-1) and fibronectin (Fn) were downregulated in post-mitotic LTC-IC. Conversely, binding and motility via VLA-5 across Fn were stimulated. No changes occurred in LTC-IC interactions with intercellular adhesion molecule-1 (ICAM-1) or with E- or P-selectin. Proliferation of uncultured LTC-IC was inhibited by VLA-4-mediated binding to VCAM-1 and the CS-1 domain of Fn, as well as binding to P-selectin. Growth of *ex vivo*-generated LTC-IC became unresponsive to these 3 ligands but was suppressed through VLA-5 engagement by the cell binding domain of Fn.

Interpretations and Conclusions. The generation of LTC-IC in expansion culture is associated with functional alterations of adhesion receptors, modulating not only binding and migration in the BM but also responsiveness to adhesion-mediated growth inhibitory signals. Such changes may limit homing and engraftment of expanded primitive stem/progenitor cells.

Key words: hematopoietic stem cells, cell culture, cell adhesion, chemotaxis, cell division, bone marrow.

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There is considerable interest in *ex vivo* expansion procedures to improve the efficiency and broaden the clinical use of hematopoietic stem cell (HSC) transplantation. Although large amplification of committed progenitors is readily achieved under current *ex vivo* culture protocols, only modest expansion of transplantable stem cells has been reported.[1,2] Several lines of evidence indicate that HSC execute self-renewal divisions and therefore expand in culture using optimal combinations of early acting cytokines. Using cell tracking procedures, it was shown that the majority of non-obese diabetic severe combined immune-deficient (NOD/SCID) mice repopulating cells (SRC) harvested from short-term expansion cultures were the progeny of cells that had executed at least one division.[3] Other reports documented efficient retroviral transduction of HSC in *ex vivo* cultures[4,5] which constitutes yet more evidence of self-renewal division since vector integration occurs in post-mitotic cells. Thus, the evidence of stem cell proliferation in *ex vivo* culture must be reconciled with the absence of their quantitative expansion. It was hypothesized that proliferating stem cells lack appropriate engraftment capabilities to be detected in xenogeneic transplantation assays. In support of this view, there is evidence of cell cycle-related changes in the engraftment capacity of adult and neonatal stem cells.[6-9] Consequently, primitive hematopoietic cells harvested after self-renewal division *ex vivo* might interact differently with ligands of the bone marrow (BM) microenvironment, as compared with their uncultured counterparts. The present study was undertaken to directly examine the validity of this hypothesis. Anatomical stem cell localization is mediated by multiple BM ligands. Stem cell homing to the bone marrow involves rolling on endothelial E- and P-selectins,[10,11] firm adhesion to vascular cell

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adhesion molecule–1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), followed by transendothelial migration via very late antigen (VLA)-4 ($\alpha_4\beta_1$ integrin) binding to VCAM-1 as well as VLA-4 and VLA-5 ($\alpha_5\beta_1$) adhesion to Fn.[12] Directed migration across BM endothelial and stromal cells is dependent on the chemokine stromal-derived factor-1 (SDF-1).[13,14] Intracellular signals triggered by engagement of adhesion receptors play an important role in controlling progenitor proliferation.[15] Adhesion to fibronectin (Fn) and P-selectin inhibits the cycling of progenitor cells, through engagement of VLA-4[16] and P-selectin glycoprotein ligand-1 (PSGL-1),[17] respectively. Stimulation of adhesion receptors expressed by BM-homed cells may be critical in controlling the balance between quiescence and proliferation in the post-transplantation period.[18]

In this report, mobilized peripheral blood (PB) hematopoietic cells with long term culture-initiation potential were isolated after self-renewal division *ex vivo*, by high resolution cell tracking. Adhesion and migration interactions with stromal ligands were studied. Furthermore, the effect of adhesion on primitive progenitor cell proliferation was also determined.

Design and Methods

Hematopoietic cell isolation

After obtaining informed consent, mobilized PB samples were collected from normal adult volunteers according to the guidelines established by the Ethical Committee of the University of Liège. Mobilization was achieved by daily granulocyte-colony stimulating factor (G-CSF; Amgen, Brussels, Belgium), administration at 10 μg/kg for 5 consecutive days. Cells were collected by apheresis on day 5. PB CD133$^+$ cells were isolated by immunomagnetic selection using CD133 CliniMACS columns (Miltenyi Biotech, Bergisch Gladbach, Germany). The purity of selected cells was measured according to the manufacturer's protocol and always exceeded 95%. Cells were cryopreserved in liquid nitrogen in small aliquots in 90% fetal bovine serum (FBS; Invitrogen) and 10% dimethylsulfoxide (Sigma, Bornem, Belgium).

Short-term cultures and cell division tracking

Cell samples were rapidly thawed at 37°C, extensively washed in phosphate-buffered saline (PBS; Biowhittaker, Verviers, Belgium) supplemented with 1% calf serum (CS; Biowhittaker) and resuspended in 1 mL solution of 5 μmol/L carboxy fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) in PBS. After a 10-minute incubation at 37°C, cell staining was stopped by the addition of 9 mL ice-cold PBS 20% FBS. Cells were washed twice and resuspended in Iscoves' medium (Biowhittaker) supplemented with 20% BIT serum substitute (Stem Cell Technologies, Meylan, France) before undergoing a preparative sort. This consisted in selecting a homogeneous population of CFSE-stained cells using a narrow gate of 40-channel width in order to increase subsequent resolution of cell divisions, as previously described.[3]

CFSE-stained CD133$^+$ cells were used as such in downstream assays of long-term culture-initiating cells (LTC-IC) adhesion, migration or growth inhibition (*see below*) or were plated in short-term expansion culture in Iscove's medium supplemented with 20% BIT, 2 mmol/L alanyl-glutamine, 1% (v/v) cholesterol-rich lipids, 1 mmol/L sodium pyruvate (all from Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin and 5×10^{-2} mmol/L 2-mercaptoethanol (all from Biowhittaker). In culture, cells were stimulated for 72 hours by a combination of 100 ng/mL each of stem cell factor (SCF), thrombopoietin (TPO; both from Amgen) and flt-3 ligand (FL; Peprotech, Boechout, Belgium) and 20 ng/mL of interleukin-6 (IL-6; Amgen) and G-CSF. To identify undivided cells, 50 ng/mL colcemid (Sigma) were added to control cultures to block cell division. Cells that had undergone 1 or 2 divisions were sorted according to the dilution of CFSE staining, using gates separated by at least 50 channels to prevent cross-contamination. The purity of sorted fractions was always >90%. One and 2-division LTC-IC were characterized in adhesion, migration and proliferation inhibition assays as described below.

LTC-IC adhesion assays

Purified ligands were coated in 12-well plates at the following concentrations: fibronectin fragments CH-296 (Retronectin™), H-296 or C-274 (kindly provided by Takara Bio Inc., Otsu Shiga, Japan) at 9 μg/cm^2, human E- or P-selectin at 2 μg/cm^2, human recombinant VCAM-1 or ICAM-1 (all from R&D Systems, Abingdon, U.K.) at 1 μg/cm^2 or 2% fraction V BSA (Invitrogen, Paisley, UK) in PBS. Up to 10^5 CD133$^+$ cells were plated in Iscoves' medium 20% BIT for 1h at 37°C. Non-adherent cells were harvested by standardized washes using warm PBS 1% BSA and discarded. Adherent cells were overlayed with 60×10^3 unirradiated MS-5 cells,[19] in 1.5 mL Myelocult (Stem Cell Technologies). Control long-term cultures of input cells (adherent + non-adherent) were initiated with 1/10 of the original cell suspension plated in ligand-coated dishes in 1.5 mL Myelocult. Cultures were maintained at 33°C in 5% CO_2 with weekly changes of half the medium. After 5 weeks, cultures were trypsinized and the cell suspension was transferred in duplicate progenitor assays in a semi-solid medium consisting of 0.9% methylcellulose (Stem Cell Technologies), 30% FBS, 10% 5637 bladder carcinoma

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cell line conditioned medium, 3 U erythropoietin (Janssen-Cilag, Beerse, Belgium), 50 mmol/L 2-mercaptoethanol, 200 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in Iscoves' medium. After an additional 2-week incubation, secondary colony-forming cells, hereafter called LTC-CFC, were scored using standard criteria. The percentage of LTC-IC adhesion on each ligand was calculated, as previously described,[20] as the number of LTC-CFC harvested from long-term cultures initiated with adherent cells divided by the number of LTC-CFC produced by input cells × 10.

LTC-IC migration assays

LTC-IC migration assays were performed as previously described[20,21] in 6.5-mm diameter 5-µm pore Transwells (Costar, Elscolab, Kruibeke, Belgium). Transwell filters were coated with purified ligands as listed above. Control filters were coated with 2% fraction V BSA in PBS. Up to 10^4 CD133$^+$ cells were plated in 100 µL Iscoves' medium 20% BIT in the upper chamber of the Transwell. The bottom compartment was filled with 600 µL of conditioned medium from the murine stromal cell line MS-5 (MS-5 CM), prepared exactly as described previously.[22] After incubation at 37°C for 3 hours, the Transwell was dismantled and non-migrating cells were discarded. Migrating cells were transferred to 12-well plates seeded 24 hours previously with 60×10^3 unirradiated MS-5 cells in 1.5 mL Myelocult. Control long-term cultures of input cells (migrating + non-migrating) were initiated with 1/10 of the original cell suspension incubated in MS-5 CM for 3 hours in ligand-coated dishes and then overlayed with MS-5 cells in 1.5 mL Myelocult. LTC-IC activity was measured as described above. The percentage of LTC-IC migration across each ligand was defined as the number of LTC-CFC harvested from long-term cultures initiated with migrating cells divided by the number of LTC-CFC produced by input cells × 10.

Assessment of LTC-IC growth inhibition

CFSE-stained CD133$^+$ cells were incubated in plates coated with different purified ligands as described above, or 2% BSA as control. Cells were cultured for 7 days in complete medium supplemented with low cytokine concentrations (1 ng/mL each of SCF, TPO and FL, and 200 pg/mL each of G-CSF and IL-6). Progeny cells were overlayed with unirradiated MS-5 cells to initiate bulk LTC-IC assays. Long-term cultures and secondary progenitor assays were carried out as described above. Alternatively, CFSE-labeled CD133$^+$ cells were first stimulated in uncoated plates in complete medium supplemented with high concentrations of cytokines, i.e., 100 ng/mL each of SCF, TPO and FL and 20 ng/mL of IL-6 and G-CSF. After 3 days, 1 and 2-division cells were sorted, washed and transferred to plates coated with purified ligands or 2% BSA in low cytokine concentrations for an additional 7 days. LTC-IC output of progeny cells was assessed as described above.

Statistical analysis

Results are reported as mean ± standard error of the mean (SEM). Comparisons were made using two-tailed Student's t-tests.

Results

Self-renewal divisions of LTC-IC in short-term culture

It has been previously reported that a small but significant proportion of progenitor cells, endowed with primitive hematopoietic properties, is resistant to cytokine stimulation for up to 7 days in expansion cultures supplemented with combinations of IL-3, SCF and/or IL-6.[23,24] More recent studies using additional early acting cytokines such as FL and TPO have demonstrated that the vast majority of progenitor cells, including the most primitive subset, respond to short-term growth factor stimulation[3,25] To monitor cell divisions in asynchronously proliferating CD133$^+$ cells in culture, we used cell tracking with CFSE as depicted in Figure 1. After 72 hours in culture, 60.2±0.5, 27.9±0.6 and 3.5±0.4% of expanded cells had executed 1, 2 or 3 divisions, respectively. Undivided cells represented 8.4±0.4% of harvested cells at this time point (Figure 1B). After an additional 24 hours of culture, most of the cells had reached the 2- or 3-division stage (Figure 1C). In order to obtain maximal proportions of cells that had divided once or twice, a 72-hour culture duration was chosen for all experiments. Average increase in total cell number was 2.7 fold at this time point. One- and 2-division cells were then separated by flow cytometric cell sorting (Figure 1). LTC-IC activity of unstimulated CD133$^+$ cells was determined and compared to that of 1- and 2-division cells. Uncultured CD133$^+$ cells yielded 28.5±0.5 week 5 LTC-CFC per 100 input cells while 1- and 2-division cells produced 27.9±1.1 and 28.9±1.2 week 5 LTC-CFC per 100 input cells, respectively (n=8). Thus, LTC-IC were quickly induced to proliferate and executed self-renewal divisions in these culture conditions.

Alterations of adhesion receptor function in ex vivo-generated LTC-IC

Adhesion and migration properties of LTC-IC were determined in CFSE-stained CD133$^+$ cells without any prior cytokine stimulation and compared to those of cells having undergone 1 or 2 divisions in short-term suspension cultures. Static binding to Fn fragment CH-296 (Retronectin™), which comprises binding sites for both VLA-4 and VLA-5 integrins, was observed in

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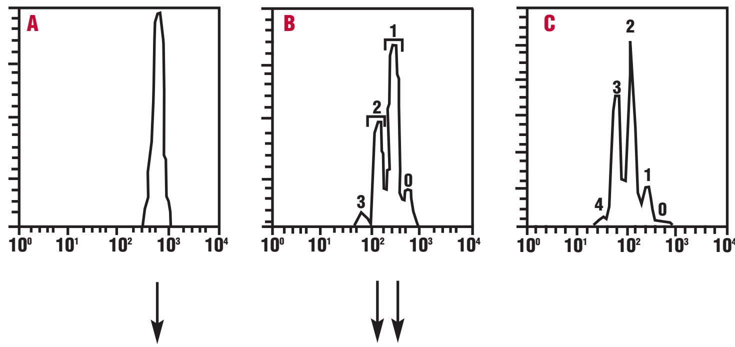

Figure 1. Cell tracking of peripheral blood CD133+ cells in expansion cultures. A. FACS profile of CFSE-stained peripheral blood CD133+ cells before culture. B. After culture for 3 days with SCF, FL, TPO, G-CSF and IL-6, successive cell generations were identified as indicated on top of each peak. A control culture in the presence of colcemid was used as reference for undivided cells. C. CFSE histogram after an additional 24 hours in culture, showing the progression of cell proliferation. Adhesion, migration and growth inhibition assays were carried out in uncultured cells and in cells having executed 1 and 2 divisions after 3 days in culture.

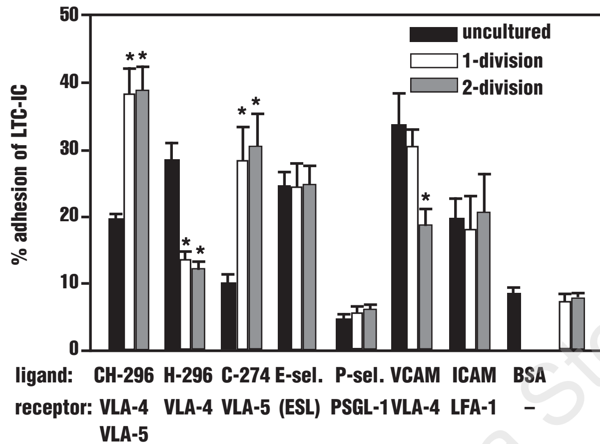

Figure 2. Changes in binding properties of LTC-IC generated *ex vivo*. LTC-IC adhesion was determined on Fn fragments CH-296, H-296 and C-274, E-selectin (E-sel.), P-selectin (P-sel.), VCAM, ICAM and BSA. Adhesion was assessed in unmanipulated LTC-IC or after 1 or 2 divisions executed in expansion cultures. *$p<0.05$ compared to adhesion of uncultured LTC-IC on the same ligand (n=4).

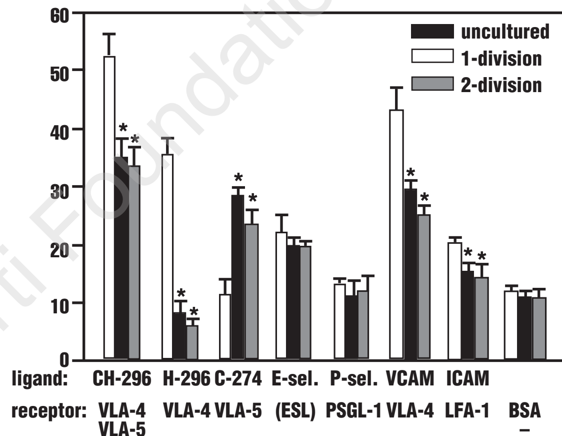

Figure 3. Migration of LTC-IC produced in *ex vivo* cultures. Migration was determined in Transwells coated with the same series of ligands as used in adhesion assays (see Figure 2), in uncultured LTC-IC or in LTC-IC harvested after 1 or 2 divisions in culture. *$p<0.05$ compared to migration of uncultured LTC-IC across the same ligand (n=4).

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19.7±0.6% of unstimulated LTC-IC (Figure 2). To distinguish the contribution of VLA-4 and VLA-5 in mediating interaction with Fn, we used Fn fragments H-296 and C-274. Using specific blocking antibodies it has been previously established that H-296 is specific for VLA-4 and C-274 is specific for VLA-5.[26,27] Unstimulated LTC-IC adhered to Fn mainly via the VLA-4 binding site as demonstrated by higher binding to Fn fragment H-296 (28.3±2.6%) than to the VLA-5 binding fragment C-274 (10±1.6% adhesion). Adhesion to immobilized E-selectin was observed in 24.3±2.5% of unstimulated LTC-IC while static adhesion to P-selectin was not detectable. Adhesion to VCAM-1 and ICAM-1 was 33.5±4.8% and 19.8±2.9%, respectively.

Activation of VLA-5 was observed for LTC-IC generated by *ex vivo* self-renewal divisions, resulting in a significant rise in adhesion capacity to C-274, both after 1 and 2 divisions ($p<0.05$, Figure 2). Conversely, VLA-4 function was downmodulated since binding of 1- or 2-division LTC-IC to H-296 and to VCAM-1 was significantly decreased ($p<0.05$). Inactivation of VLA-4 did not result in defective binding to CH-296. Finally, binding of LTC-IC to E- and P-selectin, as well as to ICAM-1 was not different in divided LTC-IC and in their uncultured counterparts.

Migration properties of *ex vivo*-generated LTC-IC

Migration of LTC-IC across stromal ligands was determined in Transwell assays in which cells were stimulated to migrate toward medium conditioned by

the stromal cell line MS-5 (MS-5 CM). We and others have previously shown that chemotaxis of primitive progenitors toward MS-5 CM is dependent on an SDF-1/CXCR-4 interaction.[21,22] Migration of unstimulated LTC-IC was optimally stimulated by CH-296 (52.8±3.63%) and VCAM-1 (43.4±4.3%) (Figure 3). Similarly to regulation of static binding to Fn, VLA-4 was more active than VLA-5 in supporting the motility of unstimulated LTC-IC since migration across H-296 (35.7±2.4%) was higher than across C-274 (11.4±2.6% migration). E-selectin (22.2±3.2%) and ICAM-1 (20.5±0.2% migration) were mildly effective in mediating motility of uncultured LTC-IC. Finally, transmigration through P-selectin did not differ significantly from control migration across BSA. Inactivation of VLA-4 in 1- and 2-division LTC-IC was demonstrated by decreased transmigration through both H-296 Fn fragment and VCAM-1 (p<0.05). Increased motility across C-274 indicated activation of VLA-5 (p<0.05). Transmigration across CH-296 was significantly reduced in divided LTC-IC. Since CH-296 carries binding sites for both VLA-4 and VLA-5, this suggests the predominant role of VLA-4 integrin in mediating LTC-IC motility across Fn. Migration through ICAM-1 was slightly reduced in divivided LTC-IC while no change occurred in LTC-IC migration through E- and P-selectins after *ex vivo* cell division (Figure 3).

The control of hematopoiesis by adhesion-mediated signals is altered in ex vivo-generated LTC-IC

Adhesion receptors are not only important for stem cell localization and trafficking but they also play a major role in regulating the hematopoietic process itself through the activation of signals influencing cell survival and growth. We investigated the effect of adhesive interactions on LTC-CFC production by uncultured and *ex vivo*-generated CD133$^+$ cells using the experimental design depicted in Figure 4. Fresh CFSE-labeled CD133$^+$ cells were cultured for 7 days onto immobilized BM ligands or control BSA and in physiological cytokine concentrations[28] after which the LTC-CFC output was assessed. Baseline LTC-CFC production after incubation in BSA-coated plates was on average 104.5±7.3 LTC-CFC per 100 cells plated at the start of the 7-day culture period. The effect of adhesion on BM ligands was expressed relative to control cultures on BSA. When cultured on CH-296 Fn fragment, LTC-CFC output was inhibited by 48±8% (p<0.05), consistently with previous reports[16] (Figure 5). The inhibition of hematopoiesis by Fn was mainly due to cell binding via VLA-4 integrin since H-296 mediated a 56±8% inhibition (p<0.05) in LTC-CFC output whereas binding to C-274 via VLA-5 had no such effect (12±3% inhibition, p>0.05). We also observed that binding to P-selectin could suppress primitive progenitor cell expansion (21±1% inhibition, p<0.05), as previously reported by Levesque and colleagues.[17] In addition, a 19±5% inhibition (p<0.05 versus control BSA cultures) in LTC-CFC output was observed when cells were incubated on VCAM-1. E-selectin and ICAM-1 had no significant effect on primitive progenitor cell development under these conditions.

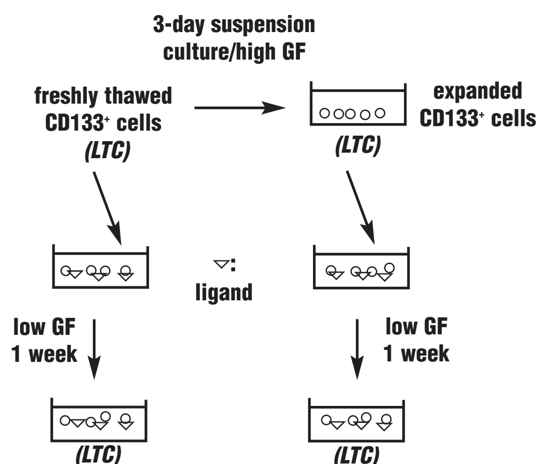

Figure 4. Control of LTC-CFC generation by BM stromal ligands: experimental design. Freshly thawed CD133$^+$ cells were plated in ligand-coated dishes and incubated for one week in medium supplemented with low growth factor (GF) concentrations (1 ng/mL each of SCF, TPO and FL, and 200 pg/mL each of G-CSF and IL-6). Alternatively, cells were stimulated for 3 days in suspension culture with high GF concentrations before being transferred to ligand-coated dishes for an additional week in low GF. LTC-CFC production ([LTC]) was measured by overlaying input CD133+ cells or their progeny with MS-5 stromal cells.

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We next investigated whether adhesion-mediated growth inhibitory signals were still operating after a prior expansion step in the absence of any adhesive ligand (Figure 4). To this end, PB CD133$^+$ cells were expanded in suspension culture for 3 days with high cytokine concentrations. At this time point, LTC-CFC output by 1- and 2-division CD133$^+$ cells (the two populations were pooled in these experiments) was 28.4±1.0 per 100 cells. The cells were then transferred to plates coated with stromal ligands or control BSA for an additional 7 days. In BSA-coated plates, LTC-CFC production increased to 96.6±3.8 per 100 input cells. In ligand-coated plates, significant alterations in adhesion-mediated suppression of hematopoiesis were observed (Figure 5). CH-296 still inhibited LTC-CFC output, although this effect was less pronounced (32±2% inhibition) than on unstimulated cells (p<0.05). Importantly, suppression of hematopoiesis by Fn in cultured LTC-IC was then mainly mediated by VLA-5 and not by VLA-4 integrin as demonstrated by the lack of effect provided by H-296 (13±6% inhibition, p<0.05) and the activation of C-274-mediated inhibition (40±1%, p<0.05 compared to undivided

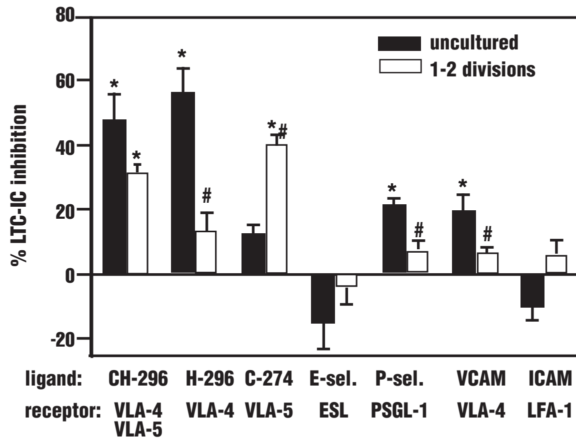

Figure 5. Inhibition of LTC-IC activity by stromal ligands before and after expansion culture. Peripheral blood CD133$^+$ cells were manipulated as described in Figure 4. Data represent % inhibition of LTC-CFC production by indicated ligands relative to cells incubated in BSA-coated plates. The effect of stromal ligands on uncultured LTC-IC or on 1- and 2-division LTC-IC was assessed (n=3). *LTC-CFC output was significantly decreased by the indicated ligand (p<0.05) as compared to cells incubated in BSA-coated plates; #inhibition of LTC-IC activity by the indicated ligand was significantly different in divided cells (p<0.05) as compared to uncultured cells.

LTC-IC). Furthemore, expanded LTC-IC became unresponsive to the inhibitory effects of P-selectin and VCAM-1.

Collectively, these data suggest that changes in the activation state of adhesion receptors in *ex vivo*-generated primitive progenitor cells not only affect their adhesion and migration capacity, but also modify their response to signals critical for the control of hematopoiesis by the bone marrow microenvironment.

Discussion

In the present study, we demonstrate that after self-renewal division in short-term culture, there were major alterations in the interactions of LTC-IC with BM stromal ligands and in their response to adhesion-mediated growth-inhibitory signals.

We observed an activation of VLA-5-mediated adhesion to Fn in 1- or 2-division LTC-IC, whereas VLA-4-mediated binding was inactivated. We previously reported a similar switch in integrin usage prior to any cell division in synchronized cultures of progenitor cells undergoing a first cell cycle transit in *ex vivo* culture.[20] Thus it appears that this functional inversion is independent of cell division in itself but is rather associated with *ex vivo* stimulation with high concentrations of mitogenic cytokines and persist at least up to 2 completed cell cycles. As expected from the adhesion assays, transmigration through the VLA-4 binding Fn fragment H-296 was downregulated after *ex vivo* division whereas migration across the VLA-5 binding Fn fragment C-274 increased in expanded LTC-IC. Binding and transmigration on VCAM-1 were significantly downregulated in *ex vivo*-generated LTC-IC, mostly after 2 divisions. This is further indication of a functional inactivation of α4 integrin. Conversely, no major changes were observed in the interactions with ICAM-1, except for a modest decrease in transmigration through ICAM-1-coated Transwells. Also, binding and transmigration across E- and P-selectins were not different in *ex vivo*-generated LTC-IC and in their unstimulated counterparts.

Given the pivotal role of adhesion to VCAM-1[29] and Fn[12,26,30] in stem cell localization, the present study supports the notion that otherwise functionnally intact primitive progenitor cells may accomplish self-renewal divisions in *ex vivo* suspension cultures but display a defective homing capacity. Our data further indicate that homing alterations may be mainly located in the final intramedullary steps of BM implantation, i.e. during stem/progenitor cell lodgment in specific endosteal niches,[30] whereas initial marrow seeding through interactions with endothelial selectins and ICAM-1 is preserved.

We also provide evidence that inhibitory signals mediated by integrin engagement are modified after LTC-IC self-renewal division *ex vivo*. It has been well established by Verfaillie and co-workers that engagement of Fn receptors suppresses hematopoietic progenitor cell proliferation.[28] In accordance with the present study, it has also been demonstrated that inhibition of progenitor cell proliferation by Fn binding is dependent on VLA-4 under low cytokine conditions.[16] Our data further demonstrate that LTC-IC obtained by *ex vivo* expansion under pharmacological concentrations of stimulatory cytokines are responsive to suppressive signals mediated by VLA-5 while VLA-4-mediated inhibition is downmodulated. Contact with VCAM-1 inhibited LTC-IC in unstimulated cells but not in expanded cells which is further evidence of VLA-4 inactivation. It has been demonstrated that inhibition of hematopoiesis by integrin engagement is due to a block in S phase entry[28] and is associated with elevated levels of cyclin-dependent kinase inhibitor p27^{KIP1}. Our data suggest that this response is elicited by engagement of VLA-4. It remains to be established whether LTC-IC inhibition mediated by VLA-5 and observed after stimulation with supraphysiological concentrations of cytokines occurs though the same signaling pathway. In conclusion, our data indicate a dissociation between self-renewal properties and maintenance of physiological interactions with the BM stroma, in primitive progenitor cells produced *in vitro*. Future efforts should be directed at optimizing

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culture conditions which drive stem cell self-renewal divisions while maintaining engraftment potential.

SH and JF performed the experiments and collected the data. RG set up the cell tracking labeling and sorting protocols. YB was responsible for primary cell harvesting and purification procedures. SH, JF, RG, YB and AG each contributed to the conception of the study, interpretation of data, preparation and review of the manuscript. AG is the author taking primary responsibility for the paper. JF and SH contributed equally to this work. The authors declare that they have no potential conflict of interest.

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