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Binding and Migration Across Fibronectin and VCAM-1 of Cycling Hematopoietic Progenitor Cells

ANDRÉ GOTHOT*, OLIVIER GIET, SANDRA HUYGEN and YVES BEGUIN

National Fund for Scientific Research, Brussels and University of Liège, Liège, Belgium

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Using different experimental approaches, it has been established that transplantability of hematopoietic/stem progenitor cells is ineffective during transit through the cell cycle. Although primitive stem cells are responsive to mitogenic stimulation in optimized *ex vivo* conditions, defective engraftment of generated cells may limit their detection in standard transplantation models as well as their use in clinical cell therapy. The activation level of adhesion receptors is modulated by stimulation of cytokine receptors via "inside-out" signaling. This prompted us to study the interactions of progenitor cells with fibronectin (Fn) in different phases of the cell cycle. We first demonstrated that adhesion to Fn was stimulated in $S/G_2 + M$ as compared to G_0/G_1, in *ex vivo* cultured $CD34^+$ cells, with a predominant usage of very late antigen (VLA)-5 over that of VLA-4. We next determined that maximal Fn binding in active phases of the cell cycle limited cell motility toward stromal cell-conditioned medium. It was also observed that VLA-4 and VLA-5 ability to mediate adhesion or migration varied independently during cell cycle transit. Finally, in synchronized progenitor cells executing a first cell cycle *ex vivo*, a reversible increase in Fn binding was associated with a reversible decrease in adhesion to vascular cell-adhesion molecule (VCAM)-1. Overall, these observations suggest that defective engraftment of cycling stem/progenitor cells may result, at least in part, from abnormal trafficking related to changes in the activation level of adhesion receptors.

Keywords: Stem cells; Transplantation; Cell cycle; Integrins

INTRODUCTION

Production of blood and lymphoid cells is carried out through a process known as clonal succession, which is based on the sequential activation and proliferation of one or a few hematopoietic stem cells (HSC). This theory has been experimentally confirmed in studies examining hematopoietic regeneration derived from genetically-marked HSC transplanted to irradiated recipients [1]. It was observed that while numerous clones contributed to marrow repopulation short term after transplant, long term reconstitution was oligoclonal. Thus in steady-state, the majority of HSC reside in a quiescent state in the G_0 phase of the cell cycle. Indeed, HSC may be isolated on the basis of mitotic quiescence by direct fractionation using rhodamine 123 [2] or pyronin Y (PY) labeling [3]. By simultaneous staining of cellular RNA and DNA with PY and Hoechst 33342, respectively, $CD34^+$ cells in G_0 or in G_1 may be isolated. Compared to G_1CD34^+ cells, G_0CD34^+ cells are characterized by a slower response to mitogenic stimuli, extensive differentiative and proliferative capacities and maintenance of hematopoietic function after cell division *ex vivo* [3,4]. Primitive hematopoietic function and mitotic quiescence are thus closely related. *In vivo* labeling with bromodeoxyuridine (BrdU) has been used to measure HSC turnover. Bradford and colleagues estimated that murine HSC divided on average every 19 days [5]. Since the vast majority of HSC are dormant at any given time, it appears that they periodically traverse the cell cycle and return to the G_0 phase. The cycling rate of HSC may increase in circumstances such as hematopoietic regeneration and ontogeny. The higher turnover of HSC during fetal development is evidenced by a similar repopulating active of mitotically quiescent and active cells isolated from fetal liver [6], whereas, in the adult, repopulating HSC segregate in the G_0 subset [7].

*Corresponding author. Address: Laboratoire d'Hématologie, Université de Liége, CHU Sart Tilman B35, 13, avenue de l'Hôpital, B-4000 Liège, Belgique. Tel.: +32-4-366-82-20. Fax: +32-4-366-75-36. E-mail: agothot@ulg.ac.be

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HSC TRANSPLANTABILITY AND CELL CYCLE

Many recent studies have established a relationship between the repopulating capacity of transplanted HSC and their position in the cell cycle. Quesenberry and colleagues have demonstrated that cell cycle activation of murine HSC was associated with impaired engraftment [8]. This observation did not result from irreversible differentiation of stimulated cells: in cell cycle-synchronized HSC, it was observed that the regenerative capacity decreased reversibly during S phase and returned to baseline levels after completion of mitosis [9]. These landmark studies were later confirmed by showing that the transplantability of proliferating HSC could be improved by prior transfer in culture conditions inducing proliferation arrest [10]. As for human stem cells, we observed a loss of severe combined immune-deficient (SCID) mice-repopulating activity when $CD34^+$ cells, initially isolated in G_0, were induced to progress in G_1 during a first division cycle *ex vivo* [7]. Eaves and coworkers determined that, within asynchronously proliferating cells executing multiple divisions *ex vivo*, SCID-repopulating activity was restricted to cells residing in G_1 [11]. In further studies, the same authors demonstrated that treatment of cycling HSC with cell cycle inhibitors improved their transplantability [12]. Overall, these studies indicate that engraftment of primitive HSC fluctuates with cell cycle transit, even prior to any cell division (Fig. 1). Mechanisms underlying these observations are currently unknown and different explanations may be considered. First, in a cell-autonomous hypothesis, it is conceivable that primitive HSC might differentiate into committed progenitors during cell cycle transit and recover their initial function after mitosis completion. Differential access of the chromatin to critical transcription factors in various phases of the cycle might account for stem/progenitor inversions and fluctuations in engraftment [13]. Second, interactions of transplanted HSC with recipient bone marrow endothelial and stromal cells might undergo cell cycle-associated variations. Experimental evidence in support of the latter hypothesis is presented below.

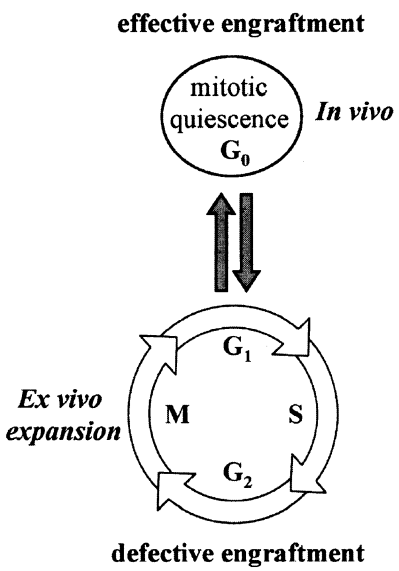

FIGURE 1 Defective engraftment of cycling stem cells. Compared to freshly isolated cells, maintained *in vivo* in G_0, proliferating stem cells harvested from *ex vivo* cultures display ineffective transplantability.

BONE MARROW IMPLANTATION OF HSC

As described for leukocyte emigration, HSC implantation into host bone marrow is carried out in several stages. During an initial margination phase, HSC leave the lumen of marrow sinusoids and roll on the surface of endothelial cells. This step is mediated by interactions of endothelial E- and P-selectins with E-selectin ligand and P-selectin glycoprotein ligand-1 expressed by HSC [14]. Activation of HSC by stromal-derived factor (SDF)-1 induces firm adhesion to endothelial vascular cell-adhesion molecule (VCAM)-1 and intercellular cell adhesion molecular (ICAM)-1 via very late antigen (VLA)-4 and lymphocyte function-associated antigen (LFA)-1 receptors, respectively. HSC transmigration through endothelial cells is supported by VLA-4/VCAM-1 and LFA-1/ICAM-1 interactions. Extravasation through the basal lamina is dependent on HSC adhesion to fibronectin (Fn) by VLA-4 and VLA-5 integrins [15]. Directional motility or chemotaxis toward extravascular marrow spaces is induced by a gradient of chemokine SDF-1 secreted by bone marrow fibroblasts [16]. SDF-1 stimulates the formation of cytoplasmic extensions, proteopodia and filipodia, which in turn, direct cell movements toward high SDF-1 concentrations. By moving across extracellular matrix proteins, such as Fn, HSC finally reach specialized stromal "niches", in which a combination of stimulatory and inhibitory factors provide homeostasis of blood cell formation. In steady-state, the pivotal role of VLA-4 in HSC retention within the bone marrow stroma has been demonstrated by Papayannopoulou and colleagues [17].

ADHESION RECEPTORS AND CELL CYCLE

Fn is recognized by two different receptors expressed by HSC, belonging to the β1 integrin family. These are heterodimeric receptors composed of a common β1 subunit and an α4 (α4β1 or VLA-4) or α5 chain (α5β1 or VLA-5). Interactions of HSC with Fn are not constitutive but highly susceptible to a variety of stimuli. Fn binding is modulated by changes in the affinity of VLA-4 and VLA-5, induced by stimulation of cytokine receptors or ligation of other adhesion molecules. Specifically, Levesque and co-workers observed that Fn binding of progenitor cells increased transiently within 30 min following *ex vivo* stimulation with stem cell factor or interleukin-3, before returning to baseline levels in 2 h [18]. Although not examined in these studies, it is unlikely that progenitor cells had progressed in the cell

cycle in such a short stimulation period. Nonetheless, these observations indicated a link between adhesion and mitogenic stimulation, which prompted us to examine Fn binding of progenitor cells during cell cycle transit. Our main model consisted in activating human $CD34^+$ cells during 48 h with stimulatory cytokines in order to obtain asynchronously proliferating cells distributed among the various phases of the cell cycle. We were able to demonstrate that Fn binding was increased in stimulated cells, specifically in cells progressing in $S/G_2 + M$. Cultured cells remaining in G_0/G_1 did not bind more efficiently to Fn than freshly isolated cells. We also determined that transit from G_0 to G_1 was associated with increased Fn adhesion [19]. These data are consistent with findings reported by Yamaguchi *et al.*, showing preferential adhesion of $CD34^+$ cells in $S/G_2 + M$ to marrow stromal cells [20]. Increased binding of cycling cells was observed not only in committed progenitors but also in primitive long-term culture-initiating cells. The role of VLA-4 and VLA-5 in mediating increased Fn binding in cycling cells was then examined. Using specific neutralizing antibodies, we observed that VLA-5 mediated adhesion of cycling cells whereas VLA-4 was more active in Fn binding of mitotically inactive cell (Fig. 2).

In spontaneously adherent cell types, such as fibroblasts or myocytes, a reciprocal relationship between adhesion and motility is well established [21]. At intermediate attachment strength, cell motility on a given substrate is achieved through labile interactions of adhesion and detachment. Conversely at maximal adhesion, the cell is immobilized and unable to respond to chemotactic stimulation. We hypothesized that enhanced adhesion to Fn in cycling progenitor cells would limit their intramedullary motility and their capacity to seed into specific niches in the bone marrow microenvironment. In *ex vivo*-stimulated $CD34^+$ cells and LTC-IC, we established that migration across Fn was higher in G_0/G_1 compared to $S/G_2 + M$. Migration of cycling and non cycling progenitor cells was similar in the absence of an adhesive substratum, indicating that adhesion strength was indeed involved in limiting cell motility [22].

The contribution of VLA-4 and VLA-5 in mediating adhesion and migration of progenitor cells was assessed in the different phases of the cell cycle. Interestingly, the functional role of the two receptors was independently modulated during cell cycle transit. VLA-5 was prominent in mediating adhesion, but not migration, of cells in $G_2 + M$. On the contrary, VLA-4 was implicated in supporting migration, but not adhesion of $G_2 + M$ $CD34^+$ cells. Thus, the reciprocal relationship between adhesion and motility is valid for independent receptors expressed by progenitor cells. Our studies establish an additional level of complexity such that this relationship fluctuates with cell cycle progression. This could induce distinct seeding pathways of HSC according to their cell cycle status and selectively promote retention and survival of non cycling cells.

In further experiments, interactions of progenitor cells with Fn, VCAM-1 and ICAM-1 were assessed during synchronized cell cycle progression [23]. Adhesion and motility of cord blood (CB) $CD34^+$ cells were assessed during progression in a first cell cycle evacuated in *ex vivo* cultures after synchronization with aphidicolin treatment [24]. Consistently with previous findings in asynchronously proliferating cells, we observed a reversible increase in Fn binding during S phase. Conversely, adhesion to VCAM-1 was reduced in S phase before returning to baseline levels after cell cycle completion. Interestingly, such fluctuation in integrin activity was not observed in $CD34^+$ cells maintained *ex vivo* in G_0/G_1 in cytokine-free conditions, which further supports the conclusion that adhesion to Fn and VCAM-1 were regulated by cell cycle-associated mechanisms (Fig. 3). Given the pivotal role of VCAM-1 in mediating transendothelial migration and intramedullary retention [25], these studies may underlie the ineffective transplantability of proliferating HSC. Transient enhancement of Fn binding might also hamper chemotaxis toward specialized stromal cells. Consistent with the latter hypothesis is the recent report that β1 integrin activation by TS2/16 anitbody abrogates the SCID-reconstituting ability of CB $CD34^+$ cells while increasing their adhesion to Fn [26].

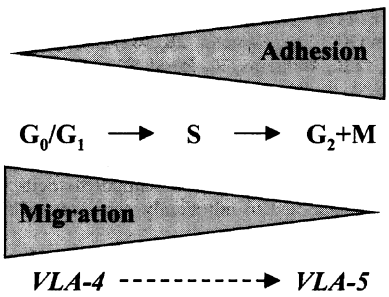

FIGURE 2 Increased adhesion and ineffective migration across Fn in cycling progenitor cells. Inverse relationship between adhesion and migration capability of cultured $CD34^+$ cells observed during transit through the cell cycle. Functional activation of VLA-5 is associated with inactivation of VLA-4 in cycling cells.

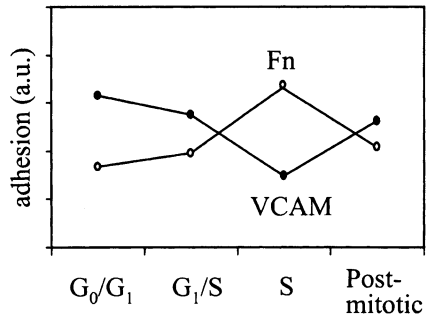

FIGURE 3 Adhesion to Fn and VCAM-1 in synchronized $CD34^+$ cells. During transit through a single cell cycle, adhesion to Fn and VCAM-1 vary in opposite directions and in a reversible fashion (a.u., arbitrary units).

REGULATION OF HEMATOPOIESIS BY SIGNALING THROUGH ADHESION RECEPTORS

Survival, proliferation and differentiation of stem/progenitor cells are coordinated by a wide variety of mitogenic and inhibitory cytokines and chemokines. In addition to their role in regulating HSC trafficking, adhesion receptors mediate additional signals regulating homeostasis of the hematopoietic system. Contact inhibition with stromal cells and extracellular matrix proteins is critical for the long-term maintenance of primitive progenitor cells in Dexter cultures [27]. Specifically, engagement of β1 integrins inhibits progenitor cell proliferation [28] through modulation of the expression and activity of cell cycle regulatory proteins (Fig. 4). Adhesion of $CD34^+$ cell to Fn induces S phase arrest, associated with higher expression of cyclin kinase inhibitor $p27^{kip1}$, decreased expression of cyclin E and reduced activity of cyclin-dependent kinase 2 [29]. While integrin signaling has been extensively studied in fibroblasts and other adherent cell types [30], the exact nature of transducer and adaptor proteins implicated in the inhibitory effects of integrin engagement in hematopoietic cells is only partially uncovered. Upon adhesion to Fn, actin filaments are organised in the cortical cytoskeleton. They associate in focal adhesion plaques with the cytoplasmic domain of the β1 integrin subunit as well as with adaptor molecules, such as talin, vinculin, α-actinin, and paxillin. Subsequent recruitment of focal adhesion kinases such as PYK2 may be involved in activating "inside-out" intracellular pathways modulating integrin affinity, and "outside-in" signals controlling cell survival and proliferation [31]. Understanding how changes in integrin function observed in *ex vivo* generated progenitor cells might affect their response to matrix-mediated inhibitory signals is currently under study [32]. Recent observations by Srour and co-workers suggest that, after transplantation, proliferation inhibition of BM-homed cells is a pre-requisite for their survival and subsequent regenerative capacity. In addition, while survival of seeded G_0/G_1 HSC is maintained, cell cycle arrest of their $S/G_2 + M$ counterparts may induce apoptosis [33]. Whether cell cycle-associated modulation of integrin activity underlies these observations is an interesting working hypothesis. Overall, defective engraftment of cycling HSC may result from abnormal trafficking as well as aberrant interactions with the bone marrow microenvironment.

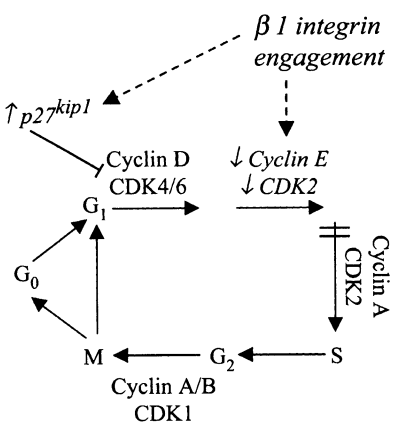

FIGURE 4 Regulation of cell cycle progression. Progression through cell cycle checkpoints is dependent on cyclin/cyclin-dependent kinase (CDK) complexes. Engagement of β1 integrins in progenitor cells results in $p27^{kip\ 1}$ upregulation, inactivation of CDK2 and cell cycle arrest [29].

CONCLUSION

Extensive progenitor cell proliferation may be achieved in short term *ex vivo* culture. Although transplantable stem cells execute self-renewal divisions in such conditions, it has not been possible to demonstrate a significant expansion of primitive HSC using SCID mouse repopulation readouts [34]. This apparent discrepancy may result from ineffective homing of cycling HSC. Analysis of HSC trafficking in combination with cell cycle status may allow the identification of defective steps in the homing process, and ultimately, provide the rationale to design *ex vivo* conditions which will maintain transplantability of *ex vivo*-generated HSC.

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