Published in final edited form as:

Exp Hematol. 2014 May ; 42(5): 404–409.e4. doi:10.1016/j.exphem.2014.01.007.

Stage specific functional roles of integrins in erythropoiesis

Tatyana Ulyanova¹, Steven M. Padilla¹, and Thalia Papayannopoulou¹

¹Department of Medicine, Division of Hematology, University of Washington, Seattle, WA, USA

Abstract

When the erythroid integrins $\alpha 5 \beta 1$ and $\alpha 4 \beta 1$ were each deleted previously at the stem cell level, they yielded distinct physiologic responses to stress by affecting erythroid expansion and terminal differentiation or only the latter, respectively. To test at what stage of differentiation the integrin effects were exerted, we created mice with $\alpha 4$ or $\alpha 5$ integrin deletion only in erythroid cells and characterized them at homeostasis and after Phenylhydrazine (PHZ)-induced hemolytic stress. The phenotype of mice with $\alpha 5$ -erythroid deletion unlike our prior data was similar to controls, especially post-stress, and these outcomes seem to reconcile divergent prior views on its role in erythropoiesis. By contrast, $\alpha 4$ integrins, whether deleted early or late, have a dominant effect on bone marrow (BM) retention of erythroblasts and on terminal erythroid maturation at homeostasis and post stress.

Introduction

In erythropoiesis integrin-dependent interactions of erythroid cells with macrophages and fibronectin in their microenvironment have been extensively studied and found to be instrumental for their proper expansion/maturation [1–4]. A host of interacting macrophage/erythroblast mediators have been uncovered [5] and references therein, but several of their partners and the signaling pathways initiated have not been elucidated. Interactions of erythroid cells with fibronectin in their microenvironment have also been found to be important for proper erythroid expansion and differentiation according to in vitro studies [2, 6]. Although $\alpha 4$ and $\alpha 5$ integrins are thought to be critically involved in these interactions [2, 6], there are conflicting data as to their roles [2, 7]. Further, whether integrins are critically involved at earlier stages of erythroid differentiation was not clear until recently. By deleting $\beta 1$ integrins in all hematopoietic cells we uncovered a major role for this integrin in erythroid responses and survival post stress, unlike the response seen by deleting

© 2014 International Society for Experimental Hematology. Published by Elsevier Inc. All rights reserved.

Corresponding Author: Thalia Papayannopoulou, M.D., University of Washington, Box 357710, Seattle, WA 98195, Phone: 206-543-5756, Fax: 206-543-3560, thalp@uw.edu.

Authorship Contribution: T.U. performed experiments, wrote part of the paper and made the figures; S.M.P. performed the experiments; T.P. designed and supervised the research and wrote the paper.

The authors declare no competing financial interests.

The online version of the article contains a data supplement.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

only $\alpha 4$ integrins [5, 8]. As mainly $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrin heterodimers are present in erythroid cells, the data implied a role for $\alpha 5$ integrin in this process distinct from the one exerted by $\alpha 4$ integrin. However, it was unclear from our previous studies [5, 8] whether deletion of all $\beta 1$ integrins was responsible for the differences and if so, at what stage of erythroid differentiation the integrin effects were exerted. To clarify integrin specific effects in erythropoiesis we created murine models with selective deletion of $\alpha 4$ or $\alpha 5$ integrins in erythroid cells and provide new data which demonstrate stage-dependent functional roles of these integrins in erythropoiesis.

Methods

Mice and treatments: to obtain mice with deletion of $\alpha 4$ or $\alpha 5$ integrins selectively in erythroid cells, we bred $\alpha 4f/f$ mice [9] or $\alpha 5f/f$ mice [10] with EpoR-Cre mice [11]. All experiments with mice were approved by the University of Washington Institutional Animal Care and Use Committee. PHZ-treatment, peripheral blood (PB) evaluation, Fluorescence Activated Cell Sorting (FACS) analysis and progenitor assays were done as described by us previously [8].

Results and Discussion

Steady state erythropoiesis in $\alpha 5f/f$ Epo-RCre⁺($\alpha 4\delta$ Ery) or $\alpha 4f/f$ Epo-RCre⁺($\alpha 5\delta$ Ery) mice

We first tested surface expression levels of $\alpha 4$ or $\alpha 5$ integrins throughout erythroid differentiation. In controls, expression of both integrins declined as erythroid cells matured; $\alpha 4$ integrin significantly declined only after the enucleation stage, whereas $\alpha 5$ declined progressively from the proerythroblast level onwards (Fig. 1A top, Suppl. Fig. 1A,B). The mean fluorescence intensity (MFI) of $\alpha 4$ at all levels is higher than $\alpha 5$ (Fig. 1B) and of interest, an $\alpha 4$ upregulation occurs at the basophilic erythroblast level in normal mice (Fig. 1B). This upregulation is of interest especially in the context of recently published data [12]. Specifically it was documented that the activation state of $\alpha 4$ integrin and its binding profile changes during erythroid maturation with the highest activation stage seen in basophilic erythroblasts. At this stage $\alpha 4\beta 1$ forms stable complexes with tetraspanins CD81, CD82, and CD151, which increase the affinity and/or clustering of $\alpha 4\beta 1$ and can bias erythroblast/macrophage interactions vs. erythroblast/fibronectin interactions by introducing different ligand-specific signaling [12].

In $\alpha 4\delta$ Ery or $\alpha 5\delta$ Ery mice, both $\alpha 4$ and $\alpha 5$ integrins were virtually deleted beyond the proerythroblast (r1) level (Fig. 1A & Suppl. Fig. 1A,B). Considering the previously reported four-fold increase of Epo-R levels from late erythroid progenitor cells to the early erythroblast BFU-e progeny [13], this pattern of ablation in our mice likely reflects more efficient Cre activation and consequent integrin ablation beyond the CD71^{hi}TER119^{lo} level. These data are consistent with maintenance of functional EpoR in late (hGlycophorinA^{hi}) erythroblasts [14]. Earlier progenitors, i.e., BFU-e/CFU-e, present in kit⁺CD71^{hi} populations, were only partially ablated (Suppl. Fig. 1B). Furthermore to directly test whether and to what extent there is deletion of integrins in early progenitors, like BFU-e and CFU-e, we cultured $\alpha 5^{+}$ vs. $\alpha 5^{-}$ BM cells from $\alpha 5\delta$ Ery mice (Suppl. Table 2) in clonogenic media. A 4-fold enrichment of both CFU-e and BFU-e was seen in the $\alpha 5^{+}$ cell fraction

(Suppl. Table 2, ~51% $\alpha 5^+$) and large BFU-e were only present in this fraction. Nevertheless in the $\alpha 5^-$ fraction (94% $\alpha 5^-$), a proportion of CFU-e/small BFU-e, was present, implying $\alpha 5$ -deletion in a proportion of these cells. These data provide useful, updated information about stage-dependent deletion using the EpoRCre mice.

At homeostasis the erythroid populations in $\alpha 5\delta$ Ery mice were similar to controls (Fig. 1C,D; Suppl. Fig. 1C,D). However in $\alpha 4\delta$ Ery mice modest deficiencies in late erythroid cells were seen in BM, not in spleen, which had abundant erythroid cells (Fig. 1C). Peripheral blood was also notable in the latter mice by a significant increase in circulating erythroblasts, but not in BFU-e or total progenitor cells (Fig. 1E,F), which is a characteristic finding in Tie2Cre⁺ $\alpha 4f/f$ mice [15]. We attribute the modest WBC increase (Suppl. Table1) and the observed differences between BM and spleen in the $\alpha 4\delta$ Ery mice to the release of erythroblasts into circulation and their subsequent accumulation and further maturation in spleen. Significant erythroblast release in PB at steady state was recently reported by conditional deletion of CD169-DTR macrophages [16], but not after clodronate use [17]. Also, in the setting of HO-1 deficiency [18], decrease in $\alpha 4$ -integrin expression was seen leading to untested speculation of premature erythroblast release in circulation. However, whether and to what extent macrophage-mediated retention [16] or other specific pathways (i.e., through Fibronectin or other $\alpha 4\beta 1$ ligands) are also involved has not been clear and needs to be explored further. Our data document that $\alpha 4$ not $\alpha 5$ integrin has a dominant effect on erythroblast retention but whether a combination of $\alpha 4$ and $\alpha 5$ enhances this effect, consistent with their combined contribution to erythroblast adhesion to fibronectin in vitro [2, 6], is unclear.

In PB a modest hematocrit (Hct) and red blood cell (RBC) reduction was seen with normal reticulocyte levels in $\alpha 4\delta$ Ery mice (Suppl. Table1). We interpret the Hct decrease as the result of suboptimal maturation of cells in BM, in which most mature forms were decreased (Fig. 1D), although a compensation by the spleen would have been anticipated. A trend towards a lower Hct with normal reticulocytes was also seen previously in Tie2Cre⁺ $\alpha 4f/f$ mice [5, 8].

Response of $\alpha 4\delta$ Ery and $\alpha 5\delta$ Ery mice to hemolytic stress

To test the response of our mice to erythroid stress, we treated them with PHZ. Kinetic responses to PHZ-induced erythroid stress are shown in Fig. 2A. A transient delay in Hct and reticulocyte increase was detected only in $\alpha 4\delta$ Ery mice. In these mice, the level of circulating erythroblasts reached new heights after PHZ-treatment, whereas only very minor increases in either control or $\alpha 5\delta$ Ery mice were seen (Fig. 2A). The expression profiles of $\alpha 4$ or $\alpha 5$ integrins in normal mice post stress again showed up-regulation of $\alpha 4$ integrin at the basophilic level (r2, Fig. 2B). In both $\alpha 4\delta$ Ery and $\alpha 5\delta$ Ery mice twice as many integrin-ablated proerythroblasts (r1) were detected compared to basal levels (Fig. 2B, red lines). This may be the result of increased EpoRCre activation (due to the early hypoxia-induced up-regulation of EpoR level) leading to a higher ablation rate and it is consistent with responses of EpoR depending on the Epo levels in the environment [19]. By contrast, unlike the picture seen at steady state, a much higher proportion of non-ablated $\alpha 4$ or $\alpha 5$ basophilic erythroblasts was seen post stress compared to proerythroblasts (Fig. 2B,C & Suppl. Fig.

2A). We speculate that these non-ablated basophilic erythroblasts are derived directly through accelerated maturation from earlier non-ablated progenitor cells with a sequestered maturation profile. These progenitors reveal themselves here through a detectable change in a surface profile. Accelerated maturation kinetics post-acute erythroid stress have been previously advocated in mice [20, 21] and also postulated in human erythropoiesis in response to acute anemia. A disordered retention of earlier markers in later cells has been the hallmark of the accelerated maturation [22, 23].

Quantitative erythropoiesis in BM or spleen post stress was also of interest. Differences in late erythroid cells were only seen in $\alpha 4\beta Ery$ mice, but unlike the data at homeostasis, these concerned both BM and spleen (Fig. 2D & Suppl. Fig. 2B,C). Such data, especially in view of elevated pretreatment erythroid cell levels in the spleen (Fig. 1C), strongly suggest impairment in optimal terminal erythroid maturation, similar to one noted previously with Tie2Cre+ $\alpha 4f/f$ mice [5, 8]. The splenic response to PHZ in $\alpha 5\beta Ery$ mice in terms of cellularity/weight was also somewhat muted (Fig. 2D & Suppl. Fig. 2C), however, it did not have an impact on the recovery kinetics (Fig. 2A). It is important to emphasize that in $\alpha 4$ - or $\alpha 5$ -deficient cells, upregulation of the alternative integrin ($\alpha 5$ in $\alpha 4$ -deficient, or $\alpha 4$ in $\alpha 5$ -deficient) was not documented (Suppl. Fig. 3).

Taken together, our data provide insightful information about the expression levels of $\alpha 4$ and $\alpha 5$ integrins during erythropoiesis, as well as about stage-dependent and Epo-R-mediated integrin ablation both at baseline and after stress, indirectly uncovering dynamic responses of Epo-R expression at these states. An upregulation of functional $\alpha 4$ integrin at basophilic stages and an indirect demonstration of accelerated erythroid maturation during stress were additional findings of interest. Further, our studies in mice with deletion of $\alpha 4\beta 1$ or $\alpha 5\beta 1$ only in erythroid cells allow for the following secure conclusions. First, $\alpha 4$ integrins have a dominant effect on erythroblast retention and on terminal erythroid maturation both at steady state and post stress. Second, $\alpha 5$ integrin is dispensable for terminal erythroid maturation in contrast to its critical influence exerted alone or in combination with other $\beta 1$ integrins deleted in all hematopoietic cells [5, 8]. Additional questions of whether a combination of $\alpha 5$ and $\alpha 4$ integrin deficiency only in erythroid cells has complementary effects and whether the signaling pathways downstream of $\alpha 4$ or $\alpha 5$ integrin triggered at each erythroid stage are distinct as we suggested [8], will require further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. R. Hynes for his generous gift of the $\alpha 5f/f$ mice, Dr. D. Engel for the EpoRCre mice and Betty Nakamoto for help with the manuscript. This research was supported by the National Institutes of Health grant DK94702.

References

1. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. *Blood*. 2008; 112:470–8. [PubMed: 18650462]
2. Eshghi S, Vogelesang MG, Hynes RO, Griffith LG, Lodish HF. Alpha4beta1 integrin and erythropoietin mediate temporally distinct steps in erythropoiesis: integrins in red cell development. *J Cell Biol*. 2007; 177:871–80. [PubMed: 17548514]
3. Hamamura K, Matsuda H, Takeuchi Y, Habu S, Yagita H, Okumura K. A critical role of VLA-4 in erythropoiesis in vivo. *Blood*. 1996; 87:2513–7. [PubMed: 8630418]
4. Hattangadi SM, Wong P, Zhang L, Flygare J, Lodish HF. From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications. *Blood*. 2011; 118:6258–68. [PubMed: 21998215]
5. Ulyanova T, Jiang Y, Padilla S, Nakamoto B, Papayannopoulou T. Combinatorial and distinct roles of $\alpha 5$ and $\alpha 4$ integrins in stress erythropoiesis in mice. *Blood*. 2011; 117:975–85. [PubMed: 20956802]
6. Vuillet-Gaugler MH, Breton-Gorius J, Vainchenker W, Guichard J, Leroy C, Tchernia G, et al. Loss of attachment to fibronectin with terminal human erythroid differentiation. *Blood*. 1990; 75:865–73. [PubMed: 2137353]
7. Kapur R, Cooper R, Zhang L, Williams DA. Cross-talk between alpha(4)beta(1)/alpha(5)beta(1) and c-Kit results in opposing effect on growth and survival of hematopoietic cells via the activation of focal adhesion kinase, mitogen-activated protein kinase, and Akt signaling pathways. *Blood*. 2001; 97:1975–81. [PubMed: 11264161]
8. Ulyanova T, Jiang Y, Padilla SM, Papayannopoulou T. Erythroid cells generated in the absence of specific beta1-integrin heterodimers accumulate reactive oxygen species at homeostasis and are unable to mount effective antioxidant defenses. *Haematologica*. 2013; 98:1769–77. [PubMed: 23812936]
9. Scott LM, Priestley GV, Papayannopoulou T. Deletion of alpha4 integrins from adult hematopoietic cells reveals roles in homeostasis, regeneration, and homing. *Mol Cell Biol*. 2003; 23:9349–60. [PubMed: 14645544]
10. van der Flier A, Badu-Nkansah K, Whittaker CA, Crowley D, Bronson RT, Lacy-Hulbert A, et al. Endothelial alpha5 and alphav integrins cooperate in remodeling of the vasculature during development. *Development*. 2010; 137:2439–49. [PubMed: 20570943]
11. Heinrich AC, Pelanda R, Klingmuller U. A mouse model for visualization and conditional mutations in the erythroid lineage. *Blood*. 2004; 104:659–66. [PubMed: 15090451]
12. Spring FA, Griffiths RE, Mankelov TJ, Agnew C, Parsons SF, Chasis JA, et al. Tetraspanins CD81 and CD82 facilitate alpha4beta1-mediated adhesion of human erythroblasts to vascular cell adhesion molecule-1. *PLoS One*. 2013; 8:e62654. [PubMed: 23704882]
13. Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T. Erythropoietin receptor characteristics on primary human erythroid cells. *Blood*. 1991; 77:2583–90. [PubMed: 1646044]
14. Singh S, Verma R, Pradeep A, Leu K, Mortensen RB, Young PR, et al. Dynamic ligand modulation of EPO receptor pools, and dysregulation by polycythemia-associated EPOR alleles. *PLoS One*. 2012; 7:e29064. [PubMed: 22253704]
15. Priestley GV, Ulyanova T, Papayannopoulou T. Sustained alterations in biodistribution of stem/progenitor cells in Tie2Cre+ alpha4(f/f) mice are hematopoietic cell autonomous. *Blood*. 2007; 109:109–11. [PubMed: 16931623]
16. Chow A, Huggins M, Ahmed J, Hashimoto D, Lucas D, Kunisaki Y, et al. CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress. *Nat Med*. 2013; 19:429–36. [PubMed: 23502962]
17. Ramos P, Casu C, Gardenghi S, Breda L, Crielgaard BJ, Guy E, et al. Macrophages support pathological erythropoiesis in polycythemia vera and beta-thalassemia. *Nat Med*. 2013; 19:437–45. [PubMed: 23502961]
18. Cao YA, Kusy S, Luong R, Wong RJ, Stevenson DK, Contag CH. Heme oxygenase-1 deletion affects stress erythropoiesis. *PLoS One*. 2011; 6:e20634. [PubMed: 21655188]

19. Porpiglia E, Hidalgo D, Koulis M, Tzafirri AR, Socolovsky M. Stat5 signaling specifies basal versus stress erythropoietic responses through distinct binary and graded dynamic modalities. *PLoS Biol.* 2012; 10:e1001383. [PubMed: 22969412]
20. Perry JM, Harandi OF, Paulson RF. BMP4, SCF, and hypoxia cooperatively regulate the expansion of murine stress erythroid progenitors. *Blood.* 2007; 109:4494–502. [PubMed: 17284534]
21. Perry JM, Harandi OF, Porayette P, Hegde S, Kannan AK, Paulson RF. Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. *Blood.* 2009; 113:911–8. [PubMed: 18927434]
22. Luck L, Zeng L, Hiti AL, Weinberg KI, Malik P. Human CD34(+) and CD34(+)CD38(–) hematopoietic progenitors in sickle cell disease differ phenotypically and functionally from normal and suggest distinct subpopulations that generate F cells. *Exp Hematol.* 2004; 32:483–93. [PubMed: 15145217]
23. Stamatoyannopoulos G, Veith R, Galanello R, Papayannopoulou T. Hb F Production in Stressed Erythropoiesis: Observations and Kinetic Models. *Ann NY Acad Sci.* 1985; 445:188–97. [PubMed: 2409871]

Fig 1. Steady state erythropoiesis in $\alpha 4$ Ery, $\alpha 5$ Ery and control mice

A. Histograms of $\alpha 4$ and $\alpha 5$ integrin expression profiles at various stages of erythroid differentiation in normal (upper row), and $\alpha 4$ - or $\alpha 5$ -ablated mice (bottom row). Total bone marrow (BM) cells were stained with CD71, Ter119, CD49d or CD49e and CD44 antibodies and analyzed by Fluorescence Activated Cell Sorting (FACS, see Suppl. Fig 1A for gating). Proerythroblasts (r1, red line), basophilic erythroblasts (r2, cyan line), polychromatic erythroblasts (r3, green line), orthochromatic erythroblasts (r4, orange line), reticulocytes (r5, lavender line), mature red blood cells (r6, dark green line) and isotype control (r7, black line). **B.** Intensity of integrin expression (mean fluorescence intensity, MFI) at various stages of erythroid maturation. Control mice (black lines), $n=5$, $\alpha 5$ Ery mice (red lines), $n=5$, $\alpha 4$ Ery mice (blue lines), $n=4$. **C.** Percent of Ter119-positive cells among total BM (left panel) and spleen (right panel) cells, color coding as in 1B. **D.** Erythroid maturation in the BM (left) and spleen (right). Percentage of cells at each maturation stage was determined by FACS as in A, color coding as in 1A. Control mice, $n=6$; $\alpha 4$ Ery mice, $n=5$; $\alpha 5$ Ery mice, $n=4$. **E.** Levels of circulating progenitors in control ($n=10$), $\alpha 4$ Ery ($n=11$) and $\alpha 5$ Ery mice ($n=11$). Filled bars are nonerythroid (CFU-C) and hatched bars are erythroid (BFU-e). **F.** Levels of circulating erythroblasts in control ($n=28$), $\alpha 4$ Ery ($n=5$) and $\alpha 5$ Ery mice ($n=4$). * indicates significant difference over control, $p<0.05$.

Fig 2. Response of $\alpha 46Ery$, $\alpha 56Ery$ and control mice to hemolytic stress

Mice received a single injection of Phenylhydrazine (PHZ, 100 mg/kg body weight) and were either followed for up to two weeks (A) or sacrificed three days after injection, PHZ d4 (B–D). **A.** Recovery from PHZ-induced stress: hematocrit (left panel), reticulocyte (middle panel) and erythroblast levels in peripheral blood (right panel) in control (n=7), $\alpha 46Ery$ (n=4) and $\alpha 56Ery$ (n=4) mice. **B.** Comparison of integrin ablation profiles in $\alpha 46Ery$ (left upper and middle panels) and $\alpha 56Ery$ (right upper and middle panels) and control mice (lower panels) at steady state and after PHZ treatment. Total BM cells were stained with CD71, Ter119, CD49e or CD49d and CD44 antibodies and cells were gated as shown in

Fig. 1A. Proerythroblasts (r1, red line), basophilic erythroblasts (r2, cyan line), polychromatic erythroblasts (r3, green line), orthochromatic erythroblasts (r4, orange line), reticulocytes (r5, lavender line), isotype control (thin black line). **C.** $\alpha 4$ or $\alpha 5$ integrin expression (percent of positive cells and intensity [MFI] in integrin-positive cells) in BM erythroblasts at various stages of maturation three days after PHZ injection. Control mice, n=9; $\alpha 48$ Ery mice, n=9 and $\alpha 58$ Ery mice, n=5. **D.** Percent of Ter119-positive cells among total BM (left panel) and spleen (right panel) cells. In A, C and D: control mice, black lines or bars; $\alpha 48$ Ery mice, blue lines or bars; $\alpha 58$ Ery mice, red lines or bars; * indicates significant difference over control, $p < 0.05$.