Absence of natural intracellular retinoids in mouse bone marrow cells and implications for PML-RARA transformation

Blood Cancer Journal (2015) 5, e284; doi:10.1038/bcj.2015.2; published online 27 February 2015

The X-RARA fusion proteins have reduced sensitivity to all-trans-retinoic acid (ATRA), and have been proposed to act by decreasing retinoid-dependent transcription required for myeloid maturation.1–3 We therefore sought to determine whether maturing myeloid cells are exposed to natural retinoid ligands in vivo. Surprisingly, we detected a paucity of natural retinoids capable of transactivating RARA-dependent transcription in adult mouse bone marrow cells, and the trace activity we observed tended to be in erythroid-lineage cells, rather than in myeloid-progenitors. This suggests that the resistance to retinoid-mediated transactivation likely has a limited role in X-RARA-dependent leukemogenesis because natural retinoids are largely absent during normal myeloid maturation.

The presence and distribution of natural retinoids have not been studied in adult hematopoiesis. To detect retinoids capable of transactivating RARA, we developed a UAS-GFP reporter mouse. UAS promoter sequences are recognized by the yeast Gal4 transcription factor, and are not activated by mammalian proteins (schema in Figure 1a). When the modular Gal4-DNA-binding domain is fused to the RARA ligand-binding domain, and expressed in UAS-GFP bone marrow cells, the reporter specifically detects intracellular retinoids that bind and transactivate RARA. This approach improves specificity of retinoid detection compared with alternative approaches, such as using the retinoid response element from the RARB promoter, which may respond nonspecifically to Rara/Rxra and Rarg/Rxra heterodimers, and Rxra/Rxra homodimers.

The mouse embryonic stem cell clone used to generate the UAS-GFP mouse was selected through a series of functional assays, to determine responsiveness and background of the randomly integrated transgene (Supplementary Figure 1). We observed only trace background GFP expression in UAS-GFP mice in the absence of a Gal4 fusion protein, with the exception of a small population of GFPdim lymphocytes in the peripheral blood and spleen (Supplementary Figure 2).

The UAS-GFP reporter was sensitive and specific to retinoids ex vivo. When Kit+ bone marrow cells were transduced with retrovirus expressing Gal4-RARA-ires-mCherry (Gal4-RARA-IC) or Gal4-RARG-ires-mCherry (Gal4-RARG-IC), we observed a dose-dependent response to ex vivo ATRA, with subnanomolar sensitivity (Figure 1a, Gal4-RARA-IC: EC50 0.36 ± 0.14 nM; Gal4-RARG-IC: EC50 0.16 ± 0.1 nM). This corresponds with the in vitro-measured Kd by using radiolabeled ATRA (Kd = 0.2 and 0.2 nM, respectively). Synthetic receptor-specific ligands induced receptor-specific GFP expression (Figure 1b, RARA-specific agonist: BMS757; RARG-specific agonist: BMS961). RARG binds to corepressors with lower affinity than RARA.3 Consistent with this, Gal4-RARG-IC was modestly more sensitive to ATRA than Gal4-RARA-IC (Figure 1a), and Gal4-RARG-IC induced a small increase in the background GFP expression in the absence of exogenous ligand (Figure 1b column 5).

In order to determine whether bone marrow cells are exposed to natural retinoids in vivo, and whether this correlates with specific stages of hematopoietic differentiation, we transduced UAS-GFP Kit+ cells with Gal4-RARA-IC or Gal4-RARG-IC, and transplanted these cells into lethally irradiated recipient mice (the RARG vector was included to improve sensitivity of natural retinoid detection and to validate the RARA findings). The recipient mice were then maintained on standard chow (PicoLab 20, Labdiet, St. Louis, MO, USA, which contains 15 IU/g vitamin A) and then analyzed after complete engraftment (~6 weeks post-transplant). Surprisingly, we observed only trace GFAFPdim bone marrow cells in mice transplanted with Gal4-RARA-IC, suggesting that few bone marrow cells have active intracellular retinoids, and these exist at or below the threshold of RARA transcriptional activation (Figures 1d and k). As a positive control, we treated mice with 50 or 200 μg ATRA in corn oil by gavage for 3 days (~7.5 and 30 mg/m2 per day), before analysis on day 4. We observed a dose-dependent induction in GFP expression, suggesting that...
most bone marrow cells can respond to active retinoids when they are present (Figure 1k).

Subgate analysis revealed that in the absence of exogenous ATRA, cells with trace retinoids (GFP−dim cells) correlated with erythroid-lineage cells (Ter119+CD71+), not with myeloid progenitors (Figure 2a). When mice were treated with 50 μg ATRA, retinoids were highest in myeloid progenitor (Kit+Gr1+ and Kit+CD11b+) and erythroid progenitor cells (Kit+CD71+ and Ter119+CD71+; Figure 2c). The largest difference between closely related populations was a 4.5-fold difference in the percent of GFP+ cells in progenitor cells (Lin−Kit+Sca− vs Kit+Lin−Sca+ (KLS)-enriched stem cells (Lin−Kit+Sca−; $P < 0.005$). In mice treated with 200 μg ATRA, the differences between specific progenitor compartments were blunted (1.7-fold difference between progenitor cells and KLS cells, $P < 0.001$, Figure 2e).
We observed similar results in mice transplanted with Gal4-RARG-IC. We observed very limited evidence of natural retinoids in bulk bone marrow cells in the absence of exogenous ATRA (low percentages of GFP dim cells; Figures 1h and k). Subgate analysis again found that the small population of cells with natural retinoids was biased toward populations of erythroid cells (Ter119+CD71+; Figure 2b). We again observed a difference in the percent of GFP+ cells in the progenitor cells vs KLS cells when the mice were untreated (14-fold, \( P < 0.0001 \), Figure 2b), and this was diminished with 50 \( \mu \)g ATRA treatment (1.6-fold, \( P < 0.1 \), Figure 2d), and abolished with 200 \( \mu \)g ATRA treatment (Figure 2f).

\( \text{RARA} \) is the target of at least 10 fusion proteins that lead to acute promyelocytic leukemia (APL), and \( \text{RARA} \) expression increases dramatically during myeloid maturation. \( \text{X-RARA} \) fusions have been proposed to act by reduced sensitivity to retinoid-dependent differentiation programs (a dominant negative effect). Therefore, we were surprised that the natural retinoids were largely absent during myeloid differentiation in vivo (Figures

---

**Figure 2.** Hematopoietic distribution of natural retinoids that transactivate either Gal4-RARA or Gal4-RARG. Bone marrow cells from Figure 1k were stained as indicated for subgate analysis of hematopoietic populations. (a, c and e) Mice transplanted with Gal4-RARA-IC retrovirus. (b, d and f) Mice transplanted with Gal4-RARG-IC retrovirus. Each circle or square represents the results from a separate mouse.
2a and b). Ex vivo, UAS-GFP cells transduced with Gal4-RARA-IC or Gal4-RARG-IC responded to subnanomolar concentrations of ATRA, and in vivo, a short course of 50 μg ATRA was adequate to induce GFP response in all measured hematopoietic compartments, suggesting that the system should be adequately sensitive to detect physiologically relevant concentrations of intracellular natural retinoids in vivo (Figures 1a, 2c and f).

Aldehyde dehydrogenase (ALDH) expression and activity have been correlated with hematopoietic stem cells, and ALDH is the rate limiting step in ATRA synthesis.11–13 Observations that ATRA can augment stem cell function ex vivo14,15 suggest that the stem cell-associated function of ALDH might occur through ATRA synthesis. However, our data suggest that KLS cells exist within a retinoid deplete environment, with disproportionately low intracellular retinoids, even when mice were treated with exogenous ATRA (Figures 2b and d). In vivo this likely occurs through stromal cell P450 activity, which is capable of rapidly eliminating local retinoids.11 Therefore, the stem-associated function of ALDH is not likely to be through ATRA generation.

We observed that even 200 μg of ATRA (given for short courses) was inadequate to activate retinoid-dependent transcription in all bone marrow cells in any hematopoietic compartment that we measured, and many cells remained mCherry+GFP+ (Figures 1f and j). Successful treatment of APL requires month-long courses of ATRA.14 Our data support this idea and suggest that long courses at pharmacologic doses may be necessary to efficiently activate retinoid-induced maturation within all leukemia cells. This may be especially important to adequately treat cells that reside in retinoid-deplete stromal cell niches (for example, leukemia stem cells).

The UAS-GFP reporter system has the advantage of being highly modular (which allows for the analysis of multiple nuclear receptor ligand-binding domains), but this requires retroviral expression of the Gal4-fusion protein and subsequent transplantation/engraftment. We are currently generating a Gal4-RARA transgenic mouse to circumvent this issue. In addition, because GFP induction by the Gal4-RARA fusion protein requires functional co-activators, the GFP read-out integrates both the intracellular concentration of active ligands, and the intracellular co-activator/co-repressor environment. Cell populations that lack appropriate co-activators to induce response to ligand, or that contain trans-repressive effects from other transcription factors, will exhibit diminished or absent response to ligands. Thus, the read-out integrates both local retinoid concentrations and the transcriptional sensitivity to retinoids of the intracellular environment.

This study has focused on intracellular retinoids that are present in bone marrow cells under steady-state conditions in vivo. It is unknown whether the expression of PML-RARA or other hematopoietic stimuli might alter intracellular retinoid production, and whether this might lead to a pan-bone marrow endocrine effect or to a lineage-restricted retinoid production. The erythroid progenitor bias observed (Figures 2b and d) at low doses of exogenous ATRA suggests that the erythroid-specific specification might lead to augmented intracellular retinoids, although this is largely speculative. Future studies will need to screen diverse hematopoietic stimuli to determine whether pathologic or physiologic processes induce in vivo retinoid availability in hematopoietic cells.

In conclusion, although X-RARA fusion proteins have been suggested to act by blocking retinoid-dependent transcriptional programs required for myeloid maturation, we observed a surprising paucity of natural retinoids capable of transactivating Gal4-RARA in primary mouse bone marrow cells in vivo. This suggests that these leukemic fusion proteins act predominantly through alternative mechanisms, and that retinoid-dependent transactivation likely has a limited role in normal, adult myeloid maturation.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**ACKNOWLEDGEMENTS**

This study was supported by NIH K99/R00 HL103975-03 (JS Welch). We thank the Alvin J. Siteman Cancer Center at the Washington University School of Medicine and the Barnes-Jewish Hospital in St Louis, MO, USA for the use of the Embryonic Stem Cell Core, which facilitated UAS-GFP embryonic stem cell transfection, selection and isolation, and the use of the Siteman Flow Cytometry Core, which provided flow cytometry and cell sorting service. The Siteman Cancer Center is supported in part by an NCI Cancer Center Support Grant #P30 CA91842. We thank Daniel Link and Matthew Walter for critically reading the manuscript.

H Niu, J Chacko, G Hadwiger and JS Welch
Department of Internal Medicine, Washington University School of Medicine, St Louis, MO, USA
E-mail: jwelch@dom.wustl.edu

**REFERENCES**

1 Tsi S, Bartelmez S, Sinicka E, Collins S. Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. Genes Dev 1994; 8: 2831–2841.

2 Wang YA, Shen K, Ishida Y, Wang Y, Kakizuka A, Brooks SC. Induction of murine leukemia and lymphoma by dominant negative retinoic acid receptor alpha. Mol Carcinog 2005; 44: 252–261.

3 Du C, Redner RL, Cooke MP, Lavau C. Overexpression of wild-type retinoid acid receptor alpha (RARalpha) recapitulates retinoid acid-sensitive transformation of primary myeloid progenitors by acute promyelocytic leukemia RARalpha-fusion genes. Blood 1999; 94: 793–802.

4 Allenby G, Bocquel MT, Saunders M, Kazmer S, Speck J, Rosenberger M et al. Retinoic acid receptors and retinoid X receptors: interactions with endogenous retinoids. Proc Natl Acad Sci USA 1993; 90: 30–34.

5 Rochette-Egly C, Germann P. Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). Nucl Recept Signal 2009; 7: e005.

6 De Braekeleer E, Douet-Guilbert N, De Braekeleer M. RARA fusion genes in acute promyelocytic leukemia: a review. Expert Rev Hematol 2014; 7: 347–357.

7 Welch JS, Yuan W, Levy TJ. PML-RARA can increase hematopoietic self-renewal without causing a myeloproliferative disease in mice. J Clin Invest 2011; 121: 1636–1645.

8 Storms RW, Trujillo AP, Springer JB, Shah L, Colvin OM, Luderman SM et al. Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc Natl Acad Sci USA 1999; 96: 9118–9123.

9 Ma AC, Chung ML, Liang R, Leung AY. A DEAD-sensitive aldehyde dehydrogenase regulates hematopoietic stem and progenitor cell development during primitive hematopoiesis in zebrafish embryos. Leukemia 2010; 24: 2090–2099.

10 Chute JP, Muramoto GG, Whitesides J, Colvin M, Safi R, Chao NJ et al. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. Proc Natl Acad Sci USA 2006; 103: 11707–11712.

11 Purton LE, Bernstein ID, Collins SJ. All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. Blood 2009; 95: 470–477.

12 Purton LE, Dworkin S, Olsen GH, Walkley CR, Fabb SA, Collins SJ et al. RARgamma is critical for maintaining a balance between hematopoietic stem cell self-renewal and differentiation. J Exp Med 2006; 203: 1283–1293.

13 Ghauri G, Yegnasubramanian S, Perkins B, Gucwa JL, Gerber JM, Jones RJ. Regulation of human hematopoietic stem cell self-renewal by the microenvironment’s control of retinoic acid signaling. Proc Natl Acad Sci USA 2013; 110: 16121–16126.

14 Sanz MA, Iacoboni G, Montesinos P. Conventional induction and post-remission therapy in APL: have we arrived? Best Pract Res Clin Haematol 2014; 27: 33–38.

15 Wang GG, Calvo KR, Pasillas MP, Sykes DB, Hacker H, Kamps MP. Quantitative production of macrophages or neutrophils ex vivo using conditional Hoxb8. Nat Methods 2006; 3: 287–293.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

Supplementary Information accompanies this paper on Blood Cancer Journal website (http://www.nature.com/bcj)