HEMATOPOIESIS

Consequences of GATA-1 Deficiency in Megakaryocytes and Platelets

By Paresh Vyas, Kenneth Ault, Carl W. Jackson, Stuart H. Orkin, and Ramesh A. Shivdasani

In the absence of the hematopoietic transcription factor GATA-1, mice develop thrombocytopenia and an increased number of megakaryocytes characterized by marked ultrastructural abnormalities. These observations establish a critical role for GATA-1 in megakaryopoiesis and raise the question as to how GATA-1 influences megakaryocyte maturation and platelet production. To begin to address this, we have performed a more detailed examination of the megakaryocytes and platelets produced in mice that lack GATA-1 in this lineage. Our analysis demonstrates that compared with their normal counterparts, GATA-1-deficient primary megakaryocytes exhibit significant hyperproliferation in liquid culture, suggesting that the megakaryocytopsis seen in animals is nonreactive. Morphologically, these mutant megakaryocytes are small and show evidence of retarded nuclear and cytoplasmic development. A significant proportion of platelets may be structurally abnormal.8 There is also a remarkable megakaryocytopsis in these animals, with a significant increase in megakaryocyte numbers in the spleen and bone marrow. Colony assays from yolk sac and fetal liver indicate that megakaryocyte progenitor numbers are normal but that some megakaryocyte progenitors exhibit marked hyperproliferation, producing abnormally large colonies principally composed of immature megakaryocytes similar to those seen in vivo. Ultrastructural examination of these megakaryocytes showed gross abnormalities with a small cytoplasm, excess rough endoplasmic reticulum, a reduction in the number of platelet granules, and underdeveloped and disorganized platelet demarcation membranes. Takahashi et al have also recently reported that when GATA-1 levels are reduced in vivo, heterozygous female mice display megakaryocytopsis in the spleen.16 These studies lead to the question as to how GATA-1 orchestrates megakaryocyte development. To begin to address this issue, we have performed a detailed characterization of GATA-1-deficient megakaryocytes and platelets. These studies...
more accurately define where GATA-1 is likely to act in this lineage.

MATERIALS AND METHODS

Generation of neo\(\Delta Hs\) and \(\Delta ne\)o\(\Delta Hs\) mice. Neo\(\Delta Hs\) and \(\Delta ne\)o\(\Delta Hs\) mice used in this study have been previously described.\(^8\) They were generated by a targeted deletion of an upstream region of the GATA-1 locus. The mutation removes at least one DNase I hypersensitive site (HS), which probably earmarks sequences important for GATA-1 expression. Two different lines of mutant mice were generated: neo\(\Delta Hs\), in which the PGK-neo selection cassette remains in the targeted locus, and \(\Delta ne\)o\(\Delta Hs\), in which the selection marker has been excised from the targeted locus. In neo\(\Delta Hs\), there is no detectable expression of GATA-1 in megakaryocytes, whereas in neo\(\Delta Hs\) mice, GATA-1 is expressed at a level of approximately 1% to 5% of normal.\(^8\) The megakaryocyte and platelet phenotypes of the two mutant mice are indistinguishable, but as \(\Delta ne\)o\(\Delta Hs\) mice are viable, they were used for studies involving adult mice.

Determination of primary megakaryocyte growth curves. Fetal liver cells were isolated from 15 neo\(\Delta Hs\) fetuses and 15 normal littermate controls at embryonic day 12.5 (E12.5). Single-cell suspensions from individual fetal livers were cultured in Dulbecco’s minimum essential medium (DMEM) with 10% fetal calf serum (FCS) supplements from individual fetal livers were cultured in Dulbecco’s minimum essential medium (DMEM) with 10% fetal calf serum (FCS) supplements. The total number of acetylcholinesterase-positive cells was counted in 50 \(\mu\)L.

DNA content analysis of primary megakaryocytes. Individual single-cell suspensions were made from bone marrow harvested from the femurs of eight \(\Delta ne\)o\(\Delta Hs\) mice and six wild-type littermates aged 6 to 10 weeks, and resuspended and washed once in CATCH medium (0.38% Na citrate + 10\(^{-3}\) mol/L adenosine + 2 \(\times\) 10\(^{-3}\) mol/L theophylline, in Ca\(^{2+}\)/Mg\(^{2+}\)-free Hank’s balanced solution).\(^19\) To assay DNA content from cultured cells, individual single-cell suspensions were made from E12.5 fetal livers from three GATA-1–deficient embryos and three littermate control embryos. These suspensions were cultured in 10% FCS in DMEM supplemented with 1% recombinant human Tpo tissue culture supernatant.\(^17\) A 50-\(\mu\)L quantity of the culture was taken every 2 days for cytocentrifugation and the slides assayed for acetylcholinesterase activity.\(^18\) The total number of acetylcholinesterase-positive cells was counted in 50 \(\mu\)L.

RESULTS

Growth dysregulation of GATA-1–deficient megakaryocytes. To quantitate the growth disturbance of primary GATA-1–deficient megakaryocytes, we compared the growth rates of GATA-1–deficient and normal megakaryocytes in vitro. Fetal liver cells isolated from neo\(\Delta Hs\) mice and wild-type littermates at E12.5 were expanded in liquid culture in the presence of recombinant Tpo and the number of acetylcholinesterase-positive cells was determined every 2 days. The growth curves (Fig 1) show a dramatic proliferative advantage for GATA-1–

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derived from CFU-Mk) as the source of mRNA. This was done to minimize differential patterns of gene expression being recorded solely because of differences in maturation state, rather than as a specific consequence of lack of GATA-1. GATA-1–deficient megakaryocytes showed significantly decreased mRNA expression of GPl30 (7.4-fold) and GPIbβ (3.6-fold), PF4 (3.4-fold) c-mpl (3.8-fold), and p45 NF-E2 (35-fold). Indeed, all of the markers studied showed decreased expression, albeit at different levels (Fig 3).

Platelet phenotype of GATA-1–deficient mice. Despite the marked megakaryocyte abnormalities outlined above, some platelets (~15% of normal) are produced in neo∆HS and ∆neo∆HS mice. In the following studies, we examined the ultrastructure of these platelets and sought to determine their competence in hemostasis.

Morphology of GATA-1–deficient platelets. Electron microscopy of platelets isolated from the peripheral blood of ∆neo∆HS mice shows a number of gross morphologic abnormalities (Fig 4). In addition to their larger volume, mutant platelets are uniformly spherical in shape, in contrast to the discoid shape of normal platelets. Rough endoplasmic reticulum and ribosomes are excessive. Most of the GATA-1–deficient platelets contain few platelet-specific granules or lack them entirely. There is marked heterogeneity in distribution of organelles, with some platelets harboring few organelles, while others are replete. Finally, many platelets have an increased internal canalicular membrane system. These findings are reminiscent of the ultrastructural abnormalities seen in the defective megakaryocytes from which these platelets are derived and indicate abnormal compartmentalization of organelles within GATA-1–deficient megakaryocytes before platelet release.

In surprising contrast to the mRNA expression data from GATA-1–deficient megakaryocytes, mutant platelets exhibit greater evidence of cytoplasmic maturity. This is evidenced by normal protein expression levels of GPIb, GpIIb, and c-mpl in GATA-1–deficient platelets compared with normal controls (Fig 5). This apparent discrepancy between the maturation state of GATA-1–deficient megakaryocytes and platelets leads us to speculate that the platelets may be produced by a small subset of megakaryocytes that achieves the greatest degree of maturity in the bulk population of GATA-1–deficient megakaryocytes, presumably from among those cells with a higher DNA content.

The high ribosome and RNA content of GATA-1–deficient platelets is suggestive of young platelet age.9 Taken together with the thrombocytopenia, this raises the possibility that the abnormal GATA-1–deficient platelets have an increased susceptibility to destruction. To address this, we studied platelet lifespan in four ∆neo∆HS and four normal animals. No difference in platelet survival was observed between wild-type and mutant platelets (Table 1), indicating that thrombocytopenia is due to a failure of platelet production.

Function of GATA-1–deficient platelets. Neo∆HS and ∆neo∆HS mice do not exhibit a bleeding diathesis in the environment of a controlled animal care facility. However, when subject to trauma (tail biopsy), mutant mice frequently bleed excessively compared with wild-type littermates. To evaluate the function of GATA-1–deficient platelets, we measured bleeding times in four ∆neo∆HS and six normal animals. As shown in Fig 6, bleeding times are substantially prolonged in

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**Fig 1. Proliferation profiles of normal (WT) and neo∆HS (MUT) primary megakaryocytes expanded from fetal liver cells in liquid culture in recombinant Tpo. The number of cells with acetylcholinesterase (AcChE) activity in 50-μL aliquots of the culture is shown as a function of the duration of culture. The values shown are the mean ± 1 SD from separate cultures of 15 normal and 15 mutant fetal livers.**
the mutant mice. It is helpful to compare these data with published results from Tpo-null mice, which have an equivalent degree of thrombocytopenia with functionally normal platelets and are reported to have an average bleeding time of 3 minutes. Thus, the excessively prolonged bleeding time of D\textsubscript{neo}D\textsubscript{HS} mice points to a functional platelet defect superimposed on thrombocytopenia.

We performed platelet activation studies on four D\textsubscript{neo}D\textsubscript{HS} mice and four normal control mice by measuring the appearance of P-selectin on the platelet cell surface in response to a variety of platelet agonists. This analysis showed a modest selective defect in activation of mutant platelets by either thrombin or a combination of adenosine diphosphate (ADP) and epinephrine, whereas activation by ADP alone was comparable to normal (Table 1).

**DISCUSSION**

The findings presented here indicate that megakaryocytes deficient in GATA-1 hyperproliferate, have a modal ploidy of 2N, express significantly lower levels of several megakaryocyte-specific genes, and produce large, structurally abnormal platelets. Connected with these abnormalities, platelet function is compromised in vivo and in vitro.

The combination of the colony and liquid culture data provide useful insights into the biology of the megakaryocytosis seen in neo\textsubscript{ΔHS} and Δneo\textsubscript{ΔHS} mice. In liquid culture there is a 15-fold increase in the maximal number of mutant megakaryocytes and these cells persist or are produced for more than twice as long. However, megakaryocytosis is not driven by an increased number of CFU-Mk progenitors, the frequency of which is similar in wild-type and mutant mice. We interpret the striking differences between wild-type and mutant cultures to reflect hyperproliferation of the progeny of a subset of mutant progenitors (Fig 7), which presumably give rise to the large abnormal colonies consisting of thousands of cells. There are two mutually nonexclusive possibilities for the different proliferative response in mutant and wild-type megakaryocyte progenitors. First, in the absence of GATA-1, there may be selective,
abnormal expansion of an otherwise rare pool of normal progenitors with high proliferative capacity that is distinct from the pool of normal CFU-Mk. Alternatively, the large colonies could arise from abnormal CFU-Mk progenitors that are present only in mutant animals; however, mutant animals must also have normal CFU-Mk progenitors, as normal-sized megakaryocyte colonies can be cultured from their hematopoietic cells. Interestingly, May-Grünwald-Giemsa staining of the abnormally large colonies shows cells not only with megakaryocytic morphology, but also some that are blast-like and others that resemble proerythroblasts (data not shown). Moreover, the progeny of these abnormally large colonies have a low but definite secondary replating potential, producing mainly erythroid colonies arrested at the proerythroblast stage (data not shown). Taken together, these results suggest that the hyperproliferating cell is not a normal CFU-Mk, but rather represents an earlier progenitor, perhaps one with biphenotypic potential. Similar colonies are almost never seen in cultures of normal fetal livers in Tpo.

It is interesting that GATA-1 impinges upon growth control in both the megakaryocytic and erythroid lineages. In erythroid cells, absence of GATA-1 leads to maturation arrest and premature apoptosis at the proerythroblast stage in vitro. The clear hyperproliferative response seen in megakaryocytes deficient in GATA-1 both in vitro and in vivo suggests that further analysis of the mechanisms by which GATA-1 influences cell growth and apoptosis in blood cells is likely to be rewarding.

GATA-1-deficient megakaryocytes exhibit evidence of retarded nuclear development, reflected by the modal ploidy of 2N, compared with 16N in wild-type controls. However, a population of mutant megakaryocytes does undergo endoredu-
Fig 4. Representative electron micrographs of platelets produced by megakaryocytes deficient in GATA-1. Normal mouse platelets (A) have a uniform appearance and show the typical discoid shape with a normal complement of organelles, including platelet-specific granules. In contrast, GATA-1-deficient platelets (B and C) are large, invariably round, heterogeneous, and show a paucity of organelles, especially platelet-specific granules. In particular, these platelets have an excess of rough endoplasmic reticulum (C), similar to the megakaryocytes from which they are derived. Bars in (A) and (B) are 5 μm; in (C), 1 μm.
plication and achieves higher DNA content. It is unclear whether this suggests that GATA-1 is not required for the initiation of endomitosis or that most megakaryocytes do require GATA-1 for endoreduplication, but that a subset of megakaryocytes can become polyploid through GATA-1–independent mechanisms. Regardless of the putative mechanism, we speculate that the minority of the megakaryocytes with higher DNA content produce platelets.

The promoters of many megakaryocyte-expressed genes have been shown to harbor functional GATA-1–binding sites, e.g., in GPIbα and GPIbβ, GPIIb, c-mpl, PF4, and GPIX promoters.5,32-36 This consistent finding may be interpreted to suggest that GATA-1 regulates an entire program of megakaryocyte gene expression and differentiation, much as has been suggested in the related erythroid lineage.37,38 Indeed, GATA-1–deficient megakaryocytes show decreased expression of mRNAs encoding all cell surface, cytoplasmic, and nuclear megakaryocyte–specific genes tested, including GPIbα and GPIbβ, c-mpl, p45 NF-E2, and PF4 (Fig 3) and GPIIb and GPIX (data not shown). There are two caveats to the interpretation of these results. First, differential expression of these mRNAs does not necessarily mean that they are all direct transcriptional targets of GATA-1. Rather, differential expression may simply reflect immaturity of GATA-1–deficient megakaryocytes if levels of these mRNAs normally increase late in megakaryocyte maturational independence. We specifically attempted to avoid this bias by conducting expression studies on normal-size megakaryocyte colonies derived from cultured wild-type and mutant CFU-Mk. Second, it remains formally possible that the differences in mRNA profile observed in cultured megakaryocytes may not be reflected in vivo. We further speculate that platelet release from GATA-1–deficient megakaryocytes is abnormal, as proplatelet formation in liquid culture is infrequent,40 and the rare proplatelets produced by GATA-1–

Bernard-Soulier syndrome (absence of GPIbβ expression) has been described with a deletion of the structural GPIbβ gene on one chromosome and a point mutation in a functional GATA-binding site in the GPIbβ promoter as the sole defect on the other, implying a critical role for GATA-1 in GPIbβ expression in vivo.39

Even though some platelets are produced in neoΔHS and ΔneoΔHS mice, they are abnormal in several respects. Morphologically, they are bigger, spherical, show an excess of rough endoplasmic reticulum and ribosomes, have few electron dense and α granules, and often have an excess of internal canalicular membranes. Ultrastructurally, the platelets are similar to the megakaryocytes from which they are released. These observations suggest that GATA-1 deficiency leads not only to defective organelle and granule formation, but also to abnormal compartmentalization of these structures in megakaryocytes, which is reflected in the platelets that are released. We further speculate that platelet release from GATA-1–deficient megakaryocytes is abnormal, as proplatelet formation in liquid culture is infrequent,40 and the rare proplatelets produced by GATA-1–

### Table 1. Lifespan and Activation of GATA-1–Deficient and Wild-Type Platelets

<table>
<thead>
<tr>
<th>Variable</th>
<th>Wild-Type</th>
<th>Mutant</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count (x 10^11/µL)</td>
<td>1.16 ± 0.12</td>
<td>0.18 ± 0.03</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Spontaneous activation (%)</td>
<td>23.4 ± 11.7</td>
<td>18.9 ± 4.0</td>
<td>NS</td>
</tr>
<tr>
<td>ADP activation (%)</td>
<td>33.3 ± 7.9</td>
<td>32.2 ± 6.0</td>
<td>NS</td>
</tr>
<tr>
<td>ADP + epinephrine activation</td>
<td>40.4 ± 8.4</td>
<td>23.5 ± 3.7</td>
<td>.01</td>
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<tr>
<td>(%)</td>
<td>39.2 ± 2.9</td>
<td>83.4 ± 2.7</td>
<td>.002</td>
</tr>
<tr>
<td>Thrombin activation (%)</td>
<td>91.4 ± 17.3</td>
<td>105.4 ± 20.5</td>
<td>NS</td>
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<tr>
<td>Platelet lifespan (hr)</td>
<td></td>
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<tr>
<td>Genotype of mice</td>
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<td>WT</td>
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| Platelets isolated from 4 wild-type and 4 GATA-1–deficient ΔneoΔHS adult mice were tested for in vivo lifespan and activation in response to a variety of agonists. Abbreviation: NS, not significant.

![Image](c- mpl, GP II b, and GPIb expression in platelets isolated from normal (WT) and ΔneoΔHS (MUT) adult mice. In the left panel, the same filter was sequentially used to assay expression for c-mpl, GPIIb, and c-src, whereas in the right panel a separate filter was sequentially used to detect expression of GPIb and c-src. The c-src signal was used to verify equivalent sample loading.)

![Image](c- mpl, GP II b, and GPIb expression in platelets isolated from normal (WT) and ΔneoΔHS (MUT) adult mice. In the left panel, the same filter was sequentially used to assay expression for c-mpl, GPIIb, and c-src, whereas in the right panel a separate filter was sequentially used to detect expression of GPIb and c-src. The c-src signal was used to verify equivalent sample loading.)

![Image](Fig 5. Western blot analysis of c-mpl, GPIIb, and GPIb expression in platelets isolated from normal (WT) and ΔneoΔHS (MUT) adult mice. In the left panel, the same filter was sequentially used to assay expression for c-mpl, GPIIb, and c-src, whereas in the right panel a separate filter was sequentially used to detect expression of GPIb and c-src. The c-src signal was used to verify equivalent sample loading.)

![Image](Fig 6. Bleeding times obtained from 6 normal (WT) and 4 ΔneoΔHS (MUT) adult mice. *Bleeding times ≥1,200 seconds.)

![Image](Table 1. Lifespan and Activation of GATA-1–Deficient and Wild-Type Platelets)
deficient megakaryocytes tend to be short buds, rather than the typical long filamentous structures that emanate from normal megakaryocytes.

Functionally, GATA-1–deficient platelets are also abnormal. Bleeding times in mutant animals are greatly prolonged, beyond that expected for the degree of thrombocytopenia. Platelet activation in vitro is modestly affected in response to either thrombin or a combination of ADP and epinephrine, but not to ADP alone. The latter findings suggest specific defects in the adrenergic and thrombin-induced pathways of platelet activation.

From the observations presented in this report, we present a model for the function of GATA-1 in megakaryocytes and platelets (Fig 7). In the absence of GATA-1, the majority of megakaryocytes are small and immature. These arise from hyperproliferation of megakaryocytes, probably from a small subset of progenitors, and many cells have 2N DNA content. Moreover, mature mutant megakaryocytes derived from CFU-Mk express lower levels of a number of megakaryocyte-associated mRNA transcripts. Nevertheless, a minority of megakaryocytes does undergo endoreduplication to have a ploidy profile that is comparable to normal and some of the mutant megakaryocytes are able to release platelets. Although these platelets display functional and structural abnormalities, they do express some platelet-associated proteins at apparently normal levels. We conclude that GATA-1 is required for the normal homeostasis of megakaryocyte numbers and nuclear and cytoplasmic maturation and platelet release.

It is unclear why some megakaryocytes are able to mature partially, albeit abnormally. This could occur if the mutations we have generated in mice (neoΔHS and ΔneoΔHS) are leaky with respect to GATA-1 expression in megakaryocytes, ie, a small proportion of cells is able to express GATA-1 to allow fuller differentiation. This is unlikely because we were unable to detect GATA-1 expression in megakaryocytes from neoΔHS mice by either immunofluorescence or RT-PCR. Moreover, although megakaryocytes from ΔneoΔHS mice show a low level of GATA-1 mRNA expression (1% to 5% by RT-PCR analysis), the megakaryocyte and platelet phenotype is identical in the two mutant strains. We hence favor the possibility that the partial maturation of megakaryocytes seen in neoΔHS and ΔneoΔHS mice occurs by GATA-1–independent means.

The GATA-1–deficient megakaryocyte phenotype contrasts markedly with the two other transcription factor knockouts that affect the megakaryocytic lineage. FOG was isolated as a protein that interacts physically with GATA-1 and enhances its capacity to drive megakaryocytic differentiation in the 416B myeloid cell line. Thus, it was surprising that FOG-null fetuses do not produce any megakaryocytic colonies, a defect in megakaryopoiesis that is distinct from neoΔHS and ΔneoΔHS mice. We have speculated that FOG has a GATA-1–independent function early in megakaryopoiesis, as well as a GATA-1–dependent role later in megakaryocyte maturation. NF-E2 is vital for terminal megakaryocyte maturation and platelet release. However, the megakaryocyte and platelet phenotypes between NF-E2–null and neoΔHS and ΔneoΔHS mice are also clearly distinguishable. As expression of p45 NF-E2 is significantly reduced in colonies derived from GATA-1–deficient CFU-Mk, it could be that part of the failure of these megakaryocytes to undergo proper terminal differentiation is related to this observation. It is likely that these three transcription factors regulate mutually exclusive sets of target genes, although some genes may be controlled in a complex manner requiring two or all three of these proteins.

**ACKNOWLEDGMENT**

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