INTRODUCTION

The differentiation of mature blood cell types from the multi-potential hemopoietic stem cell appears to be controlled, in part, through the lineage-specific expression of transcription factors. These factors regulate the expression of a series of downstream genes, which encode the proteins that determine the specialized structure and function of each mature blood cell type. In the erythroid cell lineage, several transcription factors have been identified, which may play important roles in regulating genes related to the terminal differentiation of the red blood cell (Orkin, 1990a). These factors include members of the GATA family of zinc finger proteins (Orkin, 1992; Tsai et al., 1989; Yamamoto et al., 1990), the NF-E2 basic region-leucine zipper protein (Andrews et al., 1993) and EKLF, a Kruppel-like zinc finger protein (Miller and Bieker, 1993), among others. Many genes expressed in erythroid cells, whose regulatory elements have been extensively studied, such as the alpha- and beta-globins, contain binding sites for several of these factors (reviewed by Orkin, 1990a,b). Thus, their regulation appears to involve combinatorial mechanisms, requiring the concerted activities of several transcription factors. Elucidating the roles of individual transcription factors in this process is a complex problem, and one that can be addressed through the production of targeted mutations in the genes encoding these factors and the analysis of their developmental consequences.

GATA-1 (Evans and Felsenfeld, 1989; Tsai et al., 1989)
belongs to a family of transcription factors (Yamamoto et al., 1990), named GATA-1 through GATA-4, which bind the WGATAR or ‘GATA’ motif, which is found in multiple copies in the regulatory elements of a large number of genes expressed specifically in erythroid cells (Orkin, 1992). Among mammalian hemopoietic cells, GATA-1 is expressed in erythroid cells (Tsai et al., 1989), megakaryocytes (Martin et al., 1990; Romeo et al., 1990), mast cells (Martin et al., 1990; Zon et al., 1991), eosinophils (Zon et al., 1993) and in multipotential myeloid progenitor cells (Crotta et al., 1990; Leonard et al., 1993; Mouthon et al., 1993; Sposi et al., 1992), but not in differentiated cells of the lymphoid, monocyte/macrophage or neutrophil lineages. Several of these hemopoietic cell types, including erythroid cells, mast cells and megakaryocytes, also express substantial levels of GATA-2, which can apparently bind to many of the same GATA sites as can GATA-1 (Ko and Engel, 1993; Merika and Orkin, 1993). Therefore, GATA-1 and GATA-2 may play overlapping roles in the regulation of gene expression in a particular cell type (Leonard et al., 1993; Orkin, 1992; Yamamoto et al., 1990; Zon et al., 1991, 1993).

To begin to examine the specific importance of GATA-1 for the formation and differentiation of erythroid and other blood cell types, we produced a targeted mutation in the GATA-1 gene in embryonic stem cells (Pevny et al., 1991). As this gene is X-linked (Zon et al., 1990) and the ES cell line was male, the mutant ES cells and their descendants lacked a wild-type GATA-1 gene. Therefore, we could examine the ability of the GATA-1- ES cells to contribute to various cell types in chimeric mice, without the need to pass the mutation through the germ line (Pevny et al., 1991).

We reported that the mutant ES cells were capable of contributing extensively to many non-hemopoietic tissues and to at least a fraction of white blood cell types, but not to mature red cells in fetal or adult chimeric mice. Furthermore, fetal chimeras with more extensive ES cell contributions appeared anemic and with reduced numbers of erythroblasts in the liver, suggesting an inability of the wild-type hemopoietic cells to compensate fully for the erythropoietic deficit in mutant cells (Pevny et al., 1991). In addition to in vivo analyses, the GATA-1- cells have been studied by in vitro culture under conditions that permit extensive differentiation of hemopoietic cells (Keller et al., 1993; Wiles and Keller, 1991). These studies have confirmed the inability of the mutant cells to differentiate into mature, hemoglobinized erythroid cells (Simon et al., 1992). In the present study, we have attempted to identify the stage at which GATA-1- erythroid cells are affected, as well as to examine the requirement for GATA-1 in the megakaryocyte, mast cell, macrophage and neutrophil lineages.

MATERIALS AND METHODS

Production of chimeric mice

Wild-type ES cells of line CCE (Robertson, 1987) or GATA-1- ES cells (Pevny et al., 1991), all homozygous for gpi-ic, were injected into strain MF1 blastocysts homozygous for gpi-1a. Two independent mutant ES clones, #33 and #74 were used, and Tables 1, 2 and 3 show combined data obtained with the two clones. Injected blastocysts were transferred to the uterine horns of day 2.5 pseudopregnant females, and the embryos were recovered 7-8 days later (day 9.5 to 10.5 post coitum) for analysis of yolk sac cells, or 12 days later (day 14.5 post coitum) for analysis of fetal livers. To determine the extent of chimerism, embryos were homogenized and the cells lysed by freezing and thawing. GPI isozyme distributions were determined by electrophoresis on Titan III cellulose acetate plates (Helena Laboratories) in 0.025% Tri-glycine buffer, and staining for GPI enzyme activity (McLaren and Buehr, 1981).

In vitro culture of hemopoietic colonies from yolk sac cells

Embryos and yolk sacs were dissected in ice-cold phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) (Wong et al., 1986). The yolk sacs from 5-10 chimeric embryos were added to 1 ml/yolk sac of a freshly prepared solution of 0.1% collagenase (Sigma C2674), 20% FBS in CaCl2-free, MgCl2-free PBS, and incubated at 37°C for 3 hours with occasional shaking. 40 ml of α-MEM, 2% FBS was then added, clumps were settled for 5 minutes at room temperature, and the medium and suspended cells were pipetted off, leaving a few ml in the bottom of the tube. The cells were collected by centrifugation (5 minutes at 1000 revs/minute), washed once in α-MEM, 2% FBS, resuspended in 1-2 ml of the same medium, counted and examined to make sure that most cells were present as single cells. The yield was approximately 105 cells per yolk sac at 10.5 days post coitum.

Alpha methylcellulose mixture was from Terry Fox Laboratory (HC-3230). This mixture yields a final concentration of 0.8% methylcellulose, 30% FBS and 1% BSA after addition of 1/4 volume of cells and other components. For 10 cultures, 12 ml of alpha methylcellulose mixture was mixed with 0.12 ml of 10 mM β-mercaptoethanol, 0.12 ml of 200 mM L-glutamine, 0.12 ml of 100× Pen/Strep (Gibco), 0.0075 ml of human recombinant erythropoietin (R&D Systems, tissue culture grade, 4000 U/ml, to yield final concentration 2 U/ml), and 0.156 ml of pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCCM, Terry Fox Laboratory HC-2100, 1.3% final concentration). These components were mixed by repeated passage through an 18 gauge needle, and 1.25 ml of the mixture was added to 105 yolk sac cells in a volume of 0.25 ml, mixed in a syringe fitted with an 18 gauge needle, placed into a 35 mm Petri dish, and incubated at 37°C in a humidified atmosphere of 5% CO2 in air.

For analysis of erythroid, macrophage, neutrophil and megakaryocyte-containing colonies, colonies were scored on the sixth or seventh day of incubation, and individual colonies were removed with a micropipette. Approximately half of each colony was added to 10 μl of water and frozen for GPI isozyme analysis (Fig. 1B), and the remainder was added to 50 μl of PBS and applied to a slide by centrifugation for 5 minutes at 500 revs/minute in a Shandon cytografuge. Cells were stained with Wright-Giemsa (Baxter Diff-Quik) and examined using a Nikon microscope with a 60× oil-immersion objective. Erythroid cells were identified by their characteristic round nuclei, and their nuclear and cytoplasmic staining properties, neutrophils by their segmented nuclei and clear cytoplasm, macrophages by their small round nuclei and vacuolated cytoplasm, and megakaryocytes by their large size and multilobated nuclei. Several of these hemopoietic cell types, including erythroid cells, mast cells and other hemopoietic cells, have been studied by in vitro culture under conditions that permit extensive differentiation of hemopoietic cells (Keller et al., 1993; Wiles and Keller, 1991). These studies have confirmed the inability of the mutant cells to differentiate into mature, hemoglobinized erythroid cells (Simon et al., 1992). In the present study, we have attempted to identify the stage at which GATA-1- erythroid cells are affected, as well as to examine the requirement for GATA-1 in the megakaryocyte, mast cell, macrophage and neutrophil lineages.

In vitro culture of mast cells from fetal livers

Livers from day 15.9 or 15.5 chimeric fetuses were disrupted to single cells by sequential passage through 18, 21 and 25 gauge needles in cold α-MEM, 5% FBS, 0.75% BSA (Jordan et al., 1990). The cells were washed twice, recovered by centrifugation for 5 minutes at 1500 revs/minute, resuspended by pipetting and settled for 5 minutes to remove clumped cells, and the top 90% of the medium, containing single cells, was saved.

Fetal liver (FL) cells were cultured in methylcellulose as described for yolk sac cells, except that each culture contained 2.5×104 cells,
and the medium contained 10% PWM-SCCM (Nakahata et al., 1982) and no erythropoietin. Colonies of several hundred mast cells, initially recognized by their characteristic colony morphology (Nakahata et al., 1982), arose after 8-10 days of culture, and were individually picked and replated into wells of a 24-well plate containing α-MEM, 20% FBS, 10% PWM-SCCM, L-glutamine, β-mercaptoethanol and Pen/Strep. When the cells in a well had increased in number to several thousand, a portion was removed and used for GPI analysis, to identify clones that were ES-derived. gpi-1c clones from chimeras produced with wild-type ES cells served as control mast cells (e.g., Fig. 5A,C), where as mutant mast cells (e.g., Fig. 5B,D) were gpi-1c derived cells from chimeras produced with GATA-1− ES cells. Mast cells were identified by staining with toluidine blue (Yam et al., 1971) or with alcin blue and safrin red (Levi-Schaffer et al., 1986). When a well became crowded, the cells were transferred individual 35 mm culture dishes. Half the medium was replaced 1-2 times per week, and the cells were periodically counted and stained with alcin/safrin. Although some of the colonies initially contained neutrophils and/or macrophages, only mast cells appeared to survive and proliferate under these conditions. After 10-12 days of liquid culture, a fraction of the cells was removed from each culture, washed in α-MEM, 20% FBS, and replated in α-MEM, 20%FBS containing 200 U/ml recombinant rat stem cell factor (SCF, Amgen), without PWM-SCCM. Two weeks later, cells were again sampled and stained with alcin/safrin.

**Analysis of megakaryocytes in chimeric fetal livers**

To determine megakaryocyte numbers in chimeric FL, FL fragments were fixed in formalin, postfixed in osmium tetroxide and embedded in Epon. Sections were cut at 1 μm and stained with toluidine blue. Megakaryocytes were identified based on their large size and characteristic multilobated nuclei, and the number of megakaryocytes per high powered field (HPF) was counted. 6-7 HPF were counted for each chimeric FL. The total number of cells per HPF was approximately 900 in both control and mutant chimeric FL.

To distinguish mutant from wild-type megakaryocytes in chimeric FL, touch preparations were examined by immunocytochemistry using anti-GATA-1 antisemur. The cut surface of the liver was touched to TESPA-treated microscope slides (Rentrop et al., 1986), which were fixed in paraformaldehyde, treated with anti-GATA-1 rat serum (a gift of J. D. Engel, Northwestern University), followed by goat anti-rat IgG fluorescein conjugate (Boehringer Mannheim). The slides were stained in situ for acetylcholinesterase (Ache) activity with 5 cells/35 mm plate, in modified McCoy’s 5A medium cultured, 10

**Platelet purification**

Blood was collected from the tail into heparinized microhematocrit tubes and centrifuged. The red cell pellet, the white cell buffy coat, and the plasma (containing platelets) were separated by scoring the tubes with a diamond pencil. Platelets were further purified of any contaminating white cells by passage through a Sepharose 2B column (Ginsberg and Plow, 1981), and concentrated by centrifugation at 3000 g for 15 minutes. To test for contamination with nucleated white blood cells, platelet preparations (as well as positive control samples containing added white blood cells) was applied to BSA-coated slides by cytocentrifugation, fixed in methanol:acetic acid (3:1), and stained with 0.1 μg/ml of Hoechst 33258 (Sigma) for 10 minutes, washed with water and examined by fluorescence microscopy.

**RESULTS**

**Failure of GATA-1− erythroid cells to differentiate beyond the proerythroblast stage**

To examine the stage of erythroid differentiation at which GATA-1− cells were blocked, we examined the in vitro differentiation of hemopoietic cells cultured from the yolk sacs of chimeric embryos produced with mutant or wild-type (line GPI-1C) ES cells. Mutant or WildType ES cells. Mast cells were blocked, we examined the in vitro differentiation of hemopoietic cells cultured from the yolk sacs of chimeric embryos produced with mutant or wild-type (line GPI-1C) ES cells. Mutant or WildType ES cells. Mast cell were identified by staining with toluidine blue (Yam et al., 1971) or with alcin blue and safrin red (Levi-Schaffer et al., 1986). When a well became crowded, the cells were transferred individual 35 mm culture dishes. Half the medium was replaced 1-2 times per week, and the cells were periodically counted and stained with alcin/safrin. Although some of the colonies initially contained neutrophils and/or macrophages, only mast cells appeared to survive and proliferate under these conditions. After 10-12 days of liquid culture, a fraction of the cells was removed from each culture, washed in α-MEM, 20% FBS, and replated in α-MEM, 20%FBS containing 200 U/ml recombinant rat stem cell factor (SCF, Amgen), without PWM-SCCM. Two weeks later, cells were again sampled and stained with alcin/safrin.

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Table 1. Numbers of ‘red’ vs ‘white’ colonies developing from chimeric yolk sac cells, and GPI-1 analysis of their origin from host embryo-derived vs ES cell-derived progenitors

<table>
<thead>
<tr>
<th>ES cells</th>
<th>No. of experiments</th>
<th>No. of chimeric yolk sacs</th>
<th>Avg. no. of colonies/100,000 cells</th>
<th>‘Red’ colonies</th>
<th>‘White’ colonies</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total no. (Avg.)</td>
<td>Colonies analyzed</td>
<td>Colonies analyzed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GPI-1a (host) (%)</td>
<td>GPI-1c (ES cells)</td>
<td>Total no. (Avg.)</td>
</tr>
<tr>
<td>Wild type</td>
<td>2</td>
<td>18</td>
<td>122±9</td>
<td>116 (47%)</td>
<td>128 (53%)</td>
</tr>
<tr>
<td>Mutant</td>
<td>6</td>
<td>44</td>
<td>137±17</td>
<td>316 (39%)</td>
<td>506 (61%)</td>
</tr>
</tbody>
</table>

Fig. 2. Comparison of erythroid cells, neutrophils, macrophages and megakaryocytes from wild-type and mutant colonies, stained with Wright-Giemsa. (A) Wild-type erythroid cells, including various stages of differentiation. Bar, 25 µm; (B) mutant erythroid cells, most of which resemble proerythroblasts; (C) wild-type neutrophils; (D) mutant neutrophils; (E) wild-type macrophages; (F) mutant macrophages; (G) wild-type megakaryocytes; (H) mutant megakaryocytes. All panels are printed at the same magnification as A.
Development of GATA-1− hematopoietic cells

The murine embryonic yolk sac contains multipotent and committed hematopoietic progenitors which, when cultured in vitro, give rise to definitive erythroid cells as well as differentiated cells of several non-erythroid lineages (Metcalfe and Moore, 1971; Wong et al., 1986). ES cells were injected into strain MF1 blastocysts, which were implanted into pseudopregnant foster mothers, and the chimeric embryos were recovered at day 9.5-10.5 post coitum (Fig. 1A). The chimeric embryos were subjected to GPI isozyme analysis to estimate the extent of ES cell contribution, which ranged from about 30% to 70%, while the pooled yolk sacs from each litter were used as a source of hematopoietic cells. Single cell suspensions prepared from the yolk sacs were cultured in vitro under conditions supporting the clonal growth of erythroid as well as various myeloid cell types. After 6 days, colonies were counted and categorized as ‘red’ or ‘white’, reflecting the presence or absence of macroscopically visible hemoglobinized cells (however, as described below, ‘white’ should not be assumed to mean ‘non-erythroid’). Colonies were picked, and one portion of each was used for GPI isozyme analysis to determine whether it originated from a host (GPI-1A) or an ES cell-derived (GPI-1C) progenitor (Fig. 1B), while the remainder of the cells were applied to a slide, stained with Wright-Giemsa and examined to identify the cell types present. While most colonies expressed only GPI-1A or GPI-1C, a few colonies expressed both isozymes, indicating that they contained both host embryo-derived and ES cell-derived cells; these colonies were excluded from the analysis. Colonies containing fewer than approximately 300 cells were also excluded, as they contained too few cells to permit GPI and cell type analysis.

In control experiments with wild-type ES cells, ‘red’ and ‘white’ colonies were approximately equal in number, and each type arose from host- or ES-derived progenitors with a similar frequency, as would be expected for embryos averaging 50% chimerism (Table 1). In experiments with GATA-1− ES cells, the number of total colonies per 10^5 yolk sac cells was unchanged from control values, indicating that the GATA-1− mutation did not affect the formation of clonogenic progenitors. However, the proportion of ‘red’ colonies was somewhat reduced (39% vs. 47%), and GPI analysis of these ‘red’ colonies showed that they were exclusively of host origin, confirming our earlier conclusion that GATA-1− cells were unable to produce mature, hemoglobinized erythroid cells.

Fig. 3. Identification of mutant megakaryocytes in chimeric fetal livers, by immunocytochemistry with an anti-GATA-1 antiserum. Fetal liver cells from wild-type fetal livers (A,D) or GATA-1− chimeric fetal livers (B,C,E,F) were stained with propidium iodide, which stains all nuclei (A-C) and with GATA-1 antiserum and FITC-conjugated secondary antibodies, which stains only cells expressing GATA-1 (D-F). D shows a wild-type megakaryocyte (arrowhead), which is positive for GATA-1, as well as many other GATA-1-positive cells with small, round nuclei, most of which are presumably erythroid cells. E and F each contain a mutant megakaryocyte, which can be identified by nuclear size and morphology in the corresponding propidium iodide-stained photographs (B,C), but fails to stain with the GATA-1 antiserum, indicating that it is mutant in origin. Bar in A, 25 μm; all panels were printed at the same magnification.
cells in vivo (Pevny et al., 1991). In contrast, an increased proportion of the ‘white’ colonies were of mutant ES cell origin (Table 1).

Microscopic examination showed that the ‘red’ colonies, which were all wild type (GPI-1A), included erythroid cells at various stages of maturation, ranging from the proerythroblast all the way to enucleated red cells (Fig. 2A). While the ‘white’ colonies of wild-type origin contained only non-erythroid cells (see below), many of the ‘white’ colonies of mutant origin were composed largely of cells resembling proerythroblasts and a few basophilic erythroblasts (Fig. 2B). Thus, while the GATA-1 mutation appears not to reduce the number of erythroid progenitors or interfere with their colony-forming potential, it seems to prevent their maturation beyond an early stage of differentiation.

**Differentiation of GATA-1− hemopoietic progenitors into macrophages, neutrophils and megakaryocytes**

Several non-erythroid cell types were observed in GATA-1− colonies, often together with immature erythroid cells, but in some cases in the absence of any erythroid cells. Neutrophils were observed in 7 of 81 colonies, macrophages in 24 of 81 colonies and megakaryocytes in 12 of 81 mutant colonies examined (Table 2). These mutant cells (Fig. 2D,F,H) were morphologically similar to the neutrophils, macrophages and megakaryocytes observed in wild-type colonies (Fig. 2C,E,G). Thus, GATA-1 appears not to be required for the in vitro morphological differentiation of these three cell types.

**Properties of GATA-1− megakaryocytes**

The lack of an apparent effect on the in vitro differentiation of megakaryocytes from yolk sac progenitors was of particular interest, since GATA-1 is normally expressed in the megakaryocyte lineage (Martin et al., 1990; Mouthon et al., 1993; Romeo et al., 1990). We therefore sought to confirm this observation through an examination of megakaryocytes formed in vivo, in the livers of chimeric fetuses produced with GATA-1− or control ES cells. Fetal liver (FL) preparations were first examined using a GATA-1 antiserum that stains the nuclei of cells expressing GATA-1. In control FL, in addition to staining the erythroid cells, which are characterized by their small, round nuclei, the antiserum labelled the nuclei of all megakaryocytes, which were recognized by their very large size and multi-lobated nuclei (Fig. 3A,B). The identification of megakaryocytes based on cell morphology was confirmed by staining similar cell preparations for acetylcholinesterase (Ache), a marker of the megakaryocyte lineage (Jackson, 1973; Long and Williams, 1982); all cells with large, multilobated nuclei were Ache+ (data not shown). However, in chimeric FL produced with mutant ES cells, most of the megakaryocytes failed to stain with the GATA-1 antiserum (Fig. 3B,C,E,F), indicating that they were of mutant origin. This confirmed our in vitro observations that the GATA-1− mutation did not prevent the formation of morphologically mature megakaryocytes.

Surprisingly, chimeric FL produced with mutant ES cells displayed a 4-fold increase in the number of mature megakaryocytes, when compared with FL from chimeras produced with control ES cells (Table 3). This suggested that the lack of GATA-1 either increased the rate megakaryocyte formation in the fetal liver, or decreased the rate of megakaryocyte turnover. As one measure of the rate of megakaryocyte formation, we determined the number of megakaryocyte colony-forming-units (CFU-mega) in mutant and control chimeric FL, by Ache staining. Neither the number of CFU-mega/10⁵ FL cells, nor the ratio of CFU-mega to total CFU was elevated in the mutant chimeric fetal livers (Table 3), suggesting that the observed increase in mature megakaryocytes was not due to an increase in the number of megakaryocyte progenitors.

To test the ability of GATA-1− megakaryocytes to give rise to platelets, we produced several adult chimeric mice using mutant or control ES cells. Platelets were purified from red and white blood cells, and the GPI isoynzyme distribution of various fractions were examined. In each of the three mutant chimeric mice examined, a GPI-1C band was detected in the purified platelet population (e.g., Fig. 4). To confirm the purity of the platelet preparation, we applied 1.5×10⁷ platelets to a slide and stained the slides with the fluorescent DNA-binding dye Hoechst 33258. No nucleated cells were detected (data not shown). These findings indicate that GATA-1− megakaryocytes are capable of terminal differentiation, contributing to the circulating platelet population. However, due to the small numbers of these mutant platelets, it was not possible to determine whether they are morphologically or functionally normal.

**Properties of GATA-1− mast cells**

Mast cells progenitors present in the yolk sac, fetal liver or adult bone marrow (Kitamura et al., 1979; Sonoda et al., 1983) can differentiate into cells resembling either mucosal or serosal mast cells when cultured under appropriate conditions (Nakano et al., 1985; Tsai et al., 1991). To test the ability of GATA-1− hemopoietic progenitors to form mast cells in vitro, we initially analyzed mast cell colonies that arose in yolk sac methylycellulose cultures after extended periods of culture (2-3 weeks). Mast cells were identified by their characteristic secretory

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**Table 2. Distribution of cell types in colonies of host or ES cell origin, from chimeric yolk sacs produced with wild-type or mutant ES cells**

<table>
<thead>
<tr>
<th>ES cells</th>
<th>GPI-1a (host origin)</th>
<th>GPI-1c (ES origin)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>eryth</td>
</tr>
<tr>
<td>Wild type</td>
<td>23</td>
<td>22</td>
</tr>
<tr>
<td>Percent of total</td>
<td>96%</td>
<td>9%</td>
</tr>
<tr>
<td>Mutant</td>
<td>60</td>
<td>51</td>
</tr>
<tr>
<td>Percent of total</td>
<td>85%</td>
<td>17%</td>
</tr>
</tbody>
</table>

eryth, erythroid; neutro, neutrophils; macro, macrophages; mega, megakaryocytes.
granules that stain with toluidine blue (Ishizaka et al., 1976), as well as by their expression of c-kit protein (Nocka et al., 1989), as detected by immunocytochemistry (data not shown). Of 33 mast cell colonies subjected to GPI analysis, 12 were derived from the host embryo, 14 were derived from mutant ES cells, while 7 were of mixed origin.

To examine the growth properties of GATA-1− mast cells, as well as their ability to differentiate into serosal-like mast cells (Tsai et al., 1991), we derived clonal cultures of GATA-1− and wild-type mast cells. Cells from chimeric fetal livers were plated in methylcellulose medium under conditions optimized for mast cell colony formation (Nakahata et al., 1982), and individual mast cell colonies were isolated and re-plated in liquid cultures, where they proliferated extensively for several weeks. Mutant and wild-type clonal mast cell cultures were identified by GPI analysis, and their growth rates and staining properties were compared after culture in the presence of either pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCCM), which stimulates the formation of mast cells that most resemble mucosal mast cells, or stem cell factor (SCF), which stimulates differentiation into cells resembling serosal mast cells (Nakano et al., 1985; Tsai et al., 1991). GATA-1− and wild-type mast cells grew at similar rates (data not shown), and both contained only alcian blue-positive granules after growth in PWM-SCCM (Kitamura, 1989; Nakano et al., 1985). In addition, the cells developed numerous safrin red-positive granules, characteristic of serosal mast cells, after growth in SCF (Tsai et al., 1991) (Fig. 5). Thus, by the criteria of growth rates and histochemical staining after culture under conditions that induce the differentiation of two mast cell subtypes, we were unable to observe any differences between GATA-1− and wild-type fetal liver-derived mast cells.

### DISCUSSION

In this study, we have investigated the effects of a targeted mutation in the gene for transcription factor GATA-1 on the development of several hemopoietic cell lineages. These included three lineages (erythroid, megakaryocyte and mast cell) in which GATA-1 is normally expressed during cell differentiation, as well as two lineages (macrophage and neutrophil) that arise from GATA-1-positive multipotential progenitors, but in which this factor appears to be absent during terminal differentiation. Because it has not been possible to pass the GATA-1− mutation through the germ line (Pevny et al., 1991), we based our studies on the analysis of chimeric mouse embryos and fetuses produced by injecting mutant ES cells into normal blastocysts. We were able to distinguish the ES-derived cells from the host-embryo derived cells in such chimeras through clonal analysis of cells cultured in vitro from the yolk sac, combined with the use of GPI isozyme analysis.

Our results indicate that hemopoietic cells lacking GATA-1 are able to enter the erythroid lineage, but cease differentiation at an early stage. As reported previously (Pevny et al., 1991), chimeric fetal livers produced with mutant ES cells contained reduced numbers of advanced (i.e., orthochromatic and polychromatic) erythroblasts with darkly staining, pyknotic nuclei. This observation suggested that GATA-1− cells were blocked at an earlier stage, but it did not identify the specific stage(s) at which the cells were arrested. In the present study, we found that the total number of yolk sac hemopoietic progenitors was unaffected by the genotype of the ES cells (mutant or wild type) used to produce the chimeras. However, none of the colonies derived from GATA-1− progenitors contained mature erythroid cells. Instead, many of these colonies contained cells that appeared, based on size, nuclear morphology and staining properties to be erythroid cells at an early stage of differentiation. Most of these cells resembled proerythroblasts, a few resembled basophilic erythroblasts, but no orthochromatic erythroblasts or enucleated forms were observed. This observation is consistent with the results of experiments in which GATA-1− ES cells were allowed to differentiate entirely in vitro, in which proerythroblast-like cells were also observed (Simon et al., 1992). However, in that study it could not be determined whether the development of mutant cells to the proerythroblast stage was a rare event, or the predominant fate of mutant erythroid cells; in the present studies, the conservation of the number of erythroid progenitors in mutant chimeric yolk sacs suggests that the GATA-1− mutation does not interfere with the development of the erythroid lineage prior to the proerythroblast stage.

While cells of the erythroid lineage also express a second member of the GATA family, GATA-2 (Leonard et al., 1993; Orkin, 1992; Sposi et al., 1992; Yamamoto et al., 1990), whose DNA-binding specificity is believed to overlap with GATA-1
(Ko and Engel, 1993; Merika and Orkin, 1993), our results suggest that GATA-2 cannot fully substitute for GATA-1 in terminal erythroid differentiation. This could result from either quantitative or qualitative differences between the two GATA factors. GATA-1 is normally the most abundant GATA protein late in erythroid differentiation, while GATA-2 is abundant at early stages and is then down-regulated (Leonard et al., 1993). Therefore, its level may be too low to substitute for GATA-1 in terminal differentiation. Alternatively, GATA-2 may be unable to regulate the same target genes as GATA-1. In either case, our results suggest that one or more GATA-1 target genes, which normally function at the pro-erythroblast stage or thereafter, is necessary for the terminal differentiation of red blood cells. Surprisingly, an examination of the expression of several potential target genes in GATA-1−/− ES cells differentiated in vitro, has shown that several such genes continue to be expressed at approximately normal levels in the absence of GATA-1 (Weiss et al., 1994). Thus, the molecular events underlying the observed block in the morphological differentiation of mutant erythroid cells remain to be defined.

While the erythroid progenitors that we have studied originated in the yolk sacs of 10.5 day mouse embryos, whose circulating blood contains only primitive, nucleated erythrocytes, these yolk sac progenitors form definitive erythrocytes when they differentiate in vitro (Wong et al., 1986). Therefore, we believe that our results apply to the definitive rather than the primitive erythroid lineage. However, studies of in vitro hematopoiesis from GATA-1−/− ES cells have suggested that cells of the primitive erythroid lineage may be arrested at an earlier stage than those of the definitive lineage (Weiss et al., 1994).

In hemopoietic colonies derived from the chimeric yolk sacs, we observed apparently normal neutrophils and macrophages in mutant as well as wild-type colonies. The lack of an effect of the GATA-1− mutation on these lineages is consistent with the lack of GATA-1 expression in mature myeloid cells. Although GATA-1 is normally expressed in the multipotential myeloid/erythroid progenitors prior to the divergence of the neutrophil, macrophage and erythroid lineages (Crotta et al., 1990; Leonard et al., 1993; Sposi et al., 1992), this

![Image](image-url)
transient expression in multipotential progenitors is apparently not a prerequisite for the development of the neutrophil and macrophage lineages.

In addition to erythroid cells, megakaryocytes normally express GATA-1, and several genes expressed in this lineage appear to be regulated by GATA factors (Lemarchand et al., 1993; Martin et al., 1990; Mouthon et al., 1993; Ravid et al., 1991; Romeo et al., 1990). Furthermore, it has been reported that the forced expression of GATA-1 in a multipotential cell line stimulated differentiation along the megakaryocytic lineage (Visvader et al., 1992). Despite such evidence for a role of GATA-1 in this lineage, our data indicate that the GATA-1 gene is not required for the production or terminal differentiation of megakaryocytes. First, morphologically normal megakaryocytes were observed in both mutant and wild-type hemopoietic colonies grown in vitro. Second, chimeric fetal livers contained megakaryocytes carrying the GATA-1 mutation, as indicated by lack of staining with a GATA-1-specific antiserum. Finally, platelets isolated from chimeric mice produced with mutant ES cells contained small amounts of the GPI-IC isozyme, indicating that the GATA-1 ES cells contributed to the platelet population.

Surprisingly, chimeric fetal livers produced with GATA-1− ES cells contained abnormally large numbers of megakaryocytes, most of which were mutant, suggesting that the kinetics of megakaryocyte formation or destruction was altered in cells lacking GATA-1. To test whether this overabundance was due to an increased number of megakaryocyte progenitors, we measured the number of CFU-mega in the chimeric fetal livers. However, this number was somewhat decreased, and thus did not account for the increased numbers of mature megakaryocytes. Alternatively, the GATA-1 mutation might reduce the rate of platelet formation, and thus decrease the rate of megakaryocyte turnover. While platelet production by mutant megakaryocytes does not appear to be blocked, quantitation of GPI isozyme ratios is not accurate enough (due to the relative instability of the GPI-IC isozyme; (Padua et al., 1978; West, 1977) to determine whether mutant cells may be under-represented in the platelet population.

We conclude that GATA-1 is not required for the terminal cell differentiation in the megakaryocyte lineage, as it is in the erythroid lineage. Our results are consistent with the observation that GATA-1 expression is down-regulated during PMA-induced megakaryocytic differentiation of human erythroleukemia cells, which suggested that GATA-1 might not be essential for megakaryocytic differentiation (Dai and Murphy, 1993). The lack of an absolute requirement for GATA-1 suggests that GATA-2, which is also expressed in this lineage, may overlap in function with GATA-1, or may play the more important role in this lineage. However, the apparent disturbances in megakaryocyte numbers suggests that some of the functions of GATA-1 in these cells may not be replaced by GATA-2.

A third cell type in which GATA-1 is normally expressed, and in which GATA-regulated genes have been identified, is the mast cell (Martin et al., 1990; Zon et al., 1991). We observed that GATA-1− mast cell colonies developed readily from yolk sac or fetal liver cells cultured in vitro, proliferated with normal kinetics, and were indistinguishable from wild-type mast cells in the number and histochemical staining properties of their secretory granules (Ishizaka et al., 1976; Nakano et al., 1985). Both wild-type and mutant mast cells expressed c-kit (Nocka et al., 1989), and when exposed to kit ligand (SCF) they differentiated into serosal-like mast cells, based on the presence of safrinin red-positive granules (Tsai et al., 1991). As in the megakaryocyte lineage, the lack of a major effect of the GATA-1− mutation on mast cell differentiation may be due to the relatively high level of GATA-2 expression in these cells (Zon et al., 1991). However, the GATA-1− mutation does not appear to be entirely without effect on mast cells, as the expression levels of several mast cell protease genes (Gurish et al., 1992) display significant quantitative differences in mutant vs. wild-type mast cells (L. Zon and F. C., unpublished data). Furthermore, mast cells cultured in vitro from fetal livers may not reflect the diversity of mast cells in the adult animal (Galli, 1993; Kitamura, 1989), and it remains possible that a mast cell subset not represented in this in vitro system is more severely affected by the GATA-1− mutation.

In summary, these studies have examined the requirements for transcription factor GATA-1 during the formation and differentiation of murine hemopoietic cells in several lineages. We have observed that the erythroid lineage, in which GATA-1 is the predominant GATA factor, is most severely affected by the absence of GATA-1, displaying an early block in differentiation. In contrast, the mast cell and megakaryocyte lineages, in which both both GATA-1 and GATA-2 are strongly expressed, are able to complete the processes of differentiation in the absence of GATA-1. Further molecular studies will be needed to define the downstream processes in erythroid cells that are presumably dependent on GATA-1, and whose altered expression results in the observed arrest of terminal differentiation.

This work was supported by a predoctoral fellowship from the March of Dimes Birth Defects Foundation (to L. F.), and by grants from the National Institutes of Health and American Cancer Society (to F. C.).

REFERENCES


