GATA-1 as a Regulator of Mast Cell Differentiation Revealed by the Phenotype of the GATA-1<sup>low</sup> Mouse Mutant

Anna Rita Migliaccio,1,2 Rosa Alba Rana,1 Massimo Sanchez,3 Rodolfo Lorenzini,4 Lucia Centurione,1 Lucia Bianchi,5 Alessandro Maria Vannucchi,5 Giovanni Migliaccio,1,3 and Stuart H. Orkin6

1Department of Biomorphology, University G. D’Annunzio, 66100 Chieti, Italy
2Laboratory of Clinical Biochemistry, 3Laboratory of Cell Biology, and 4Laboratory of Servizio Qualità Sicurezza Animale, Istituto Superiore di Sanità, 00161 Rome, Italy
5Department of Haematology, University of Florence, 50139 Florence, Italy
6Department of Pediatric Oncology, Children’s Hospital, Dana Farber Cancer Institute, Harvard Medical School and Howard Hughes Medical Institute, Boston, MA 02115

Abstract

Here it is shown that the phenotype of adult mice lacking the first enhancer (DNA hypersensitive site I) and the distal promoter of the GATA-1 gene (neo<sub>H9004</sub>/H9004H9004 or GATA-1<sup>low</sup> mutants) reveals defects in mast cell development. These include the presence of morphologically abnormal alcian blue<sup>H11001</sup>/H11001 mast cells and apoptotic metachromatic<sup>H11002</sup>/H11002 mast cell precursors in connective tissues and peritoneal lavage and numerous (60–70% of all the progenitors) “unique” trilineage cells committed to erythroid, megakaryocytic, and mast pathways in the bone marrow and spleen. These abnormalities, which were mirrored by impaired mast differentiation in vitro, were reversed by retroviral-mediated expression of GATA-1 cDNA. These data indicate an essential role for GATA-1 in mast cell differentiation.

Key words: mast cells • GATA-1 • differentiation • commitment • progenitor cells

Introduction

Considerable progress has been made in recent years in our understanding of mast cell differentiation (1, 2). Mast cells derive from c-kit<sup>low</sup> CD34<sup>low</sup> Sca-1<sup>+</sup> progenitor cells, present in the marrow and spleen (3, 4). These give rise in vivo to c-kit<sup>high</sup> CD34<sup>high</sup> Sca-1<sup>−/−</sup> precursors characterized by extensive proliferation capacity, the presence of small cytoplasmic granules, and absent or low expression of the receptor that binds the Fc portion of the IgE antibody with high affinity (Fc<sub>H9255</sub>RI<sup>H11002</sup>; references 4 and 5). In the mouse, mast cell precursors circulate in the fetal blood (5) to populate the connective and mucosal tissues where they reside throughout adult life and mature into the respective classes of definitive connective and mucosal mast cells (1, 2), characterized by specific protease expression profiles (6). At least two classes of mast cell progenitors have been identified in the mouse on the basis of the colonies that they give rise to in culture. In fact, a minority of the mixed (erythroid [E],* megakaryocytic [Mk], and granulocytic-monocytic [GM]) colonies detected after 14 d of culture contain mast cells (7), indicating that the progenitor cells from which they derive had mast cell differentiation potential. On the other hand, colonies composed of ≈500 mast cells each, which presumably derive from unilineage progenitor cells, have also been identified after 21 d of semisolid culture stimulated with IL-3 and stem cell factor (SCF; reference 8).

The molecular mechanisms underlying mast cell differentiation are better detailed in vitro in liquid cultures stimulated with IL-3 supplemented with either a fibroblast feeder layer (9) or SCF (10, 11). Under such conditions, mononuclear cells from the marrow (and spleen) give rise to bone marrow–derived mast cells (BMMC), which phenotypically and functionally resemble mast cell precursors purified from the connective tissues of the adult animals.

Address correspondence to Anna Rita Migliaccio, Clinical Biochemistry, Istituto Superiore Santà, Viale Regina Elena 299, 00161 Rome, Italy. Phone: 39-06-4990-2690; Fax: 39-06-4938-7143; E-mail: migliar@iss.it

*Abbreviations used in this paper: BFU, burst-forming unit; BMMC, bone marrow–derived mast cells; E, erythroid; EPO, erythropoietin; GM, granulocytic-monocytic; MC-CPA, mast cell carboxypeptidase A; Mk, megakaryocytic; PGK, phosphoglycerate kinase gene; SCF, stem cell factor; TPO, thrombopoietin; TUNEL, terminal deoxy transferase uridine triphosphate nick-end labeling.
In fact, BMMC are c-kit<sup>high</sup> FceRI<sup>−</sup> cells with small alcian blue<sup>−</sup> cytoplasmic granules (12) that reconstitute connective and mucosal mast cell function when transplanted into mast cell–deficient animals (13) and mature in 21 d into berberine sulfate<sup>−</sup> FceRI<sup>−</sup> mast cells capable of taking up and releasing serotonin after IgE/anti-IgE stimulation (10, 12).

Several genes have been implicated in the control of mast cell differentiation. For some of these, specific roles have been assigned through loss of function studies in the mouse. For example, defective mast cell differentiation results from mutations in either SCF or its receptor, c-kit (14), mutation of the signal transducers Jak3 (15) and PI3K (16), mutation of the transcription factor MITF (17), encoded by the microphthalmia locus (18), and deletion of gp49B1 (19), a member of the immunoglobulin gene superfamily.

GATA-1 (for review see reference 20) is a member of a highly conserved family of zinc finger protein-encoding genes that are expressed in progenitor cells as well as in E cells, megakaryocytes, and mast cells (21–25). Genetic approaches in the mouse have been instrumental in establishing a key role for GATA-1 expression in the regulation of E, Mk, and most recently, eosinophil (26) differentiation but have not as yet demonstrated a requirement in mast cell differentiation. This is due to the fact that GATA-1 loss of function mutants are embryonically lethal whereas mast cell differentiation is better studied in adult animals. Chimeric mice derived from a mixture of normal and null embryonic stem cells contain apparently normal numbers of mast cells deriving from the GATA-1<sup>−/−</sup> clone (27, 28). In contrast, it has been recently noted that the connective tissue of the skin from heterozygote females carrying a GATA-1 expression mutation (and that survive until adulthood) contains mast cells whose granules react with alcian blue but not with berberine sulfate, suggesting defective mast cell maturation (29). However, both studies fail to identify the precise contribution of the mutant stem cell clone to mast cells differentiated in vivo, leaving the interpretation of the results uncertain. On the other hand, the deletion of GATA-2 has been reported to ablate mast cell differentiation of embryonic stem cells (30) and GATA-2, but not GATA-1, is found expressed in connective mast cells by in situ hybridization (31) and western and RNA blotting techniques (29). Indirect evidence for a functional role for GATA-1 in mast cell differentiation is provided by the demonstration that both mature connective mast cells and BMMC activate GATA-1 expression shortly after IL-3 stimulation (29) and by the presence of GATA consensus sequences in the promoter region of mast cell–specific genes such as carboxypeptidase A (MC-CPA; reference 32) and the α chain of the human IgE receptor (33). Furthermore, ectopic GATA-1 expression activates the expression of the endogenous MC-CPA gene and of the IL n-1 receptor–related T1 gene from the mast cell–specific promoters in immortalized (32) or primary mast cells (34), respectively. However, because the consensus sequences for all of the GATA proteins are similar, the functional role of GATA-1 expression in mast cell differentiation, if any, remains uncertain.

Mice generated with a targeted deletion of upstream enhancer and promoter sequences of the GATA-1 gene express a reduced level of GATA-1 (35). Such animals, designated GATA-1<sup>−/−</sup> mice, are born thrombocytopenic (36, 37) and anemic (38). The few animals that survive to adulthood recover from their anemia (39) and have a normal life span (40). The mutation affects terminal maturation of megakaryocytes by preventing their fragmentation into pro-platelets (36, 37) and of erythroblasts by increasing their apoptotic rate (39). As a consequence of these defects, the hemopoietic tissues of the GATA-1<sup>−/−</sup> mice contain high levels of E/Mk progenitor cells (36–39). Here it is shown that mast cell differentiation is also impaired in GATA-1<sup>−/−</sup> mice. The defect is similar to that in E and Mk lineages (i.e., amplification of the progenitor compartment, increased apoptotic rate at the precursor level, and defective differentiation of the mature cells) and is reversed in vitro by forced GATA-1 expression. These data provide evidence for a direct role of GATA-1 in mast cell differentiation and suggest possible similarities between the regulatory mechanism(s) of the E/Mk and mast cell differentiation pathways.

Materials and Methods

Mice. The GATA-1<sup>−/−</sup> colony was bred at the animal facilities of the Istituto Superiore di Sanità. Littermates were genotyped by PCR at birth as previously described (39, 40). WBB6F1a W/W<sup>−/−</sup> female mice were purchased from The Jackson Laboratory. All the experiments were performed with sex- and age (4–6 mo)-matched mice under protocols approved by the institutional animal care committee.

Tissue Sampling. Blood was harvested with a capillary tube from the retro-orbital plexus and cells from the peritoneum (peri toneal lavage) were collected by gently washing the peritoneal cavity with 1 ml PBS (GIBCO BRL).

Histological Analysis. Ears and dorsal skin samples were paraffin embedded according to standard procedures. Slides of consecutive sections were dewaxed, rehydrated, and stained with regular (1% in H<sub>2</sub>O) and acidified (0.02% in 0.25% glacial acetic acid) toluidine blue (Multilab) and safranin-counterstained alcian blue (kit for acid mucosubstances, pH 2.5; Bio Optica; reference 41). Cells obtained from peritoneal lavages were cytospun onto glass slides (Cytospin 3; Shandon) and stained with May-Grunwald/ Giemsa (Sigma-Aldrich) or alcian blue. Samples for immunohistochemistry and immunofluorescence analysis of GATA-1 expression were fixed with 2.5% glutaraldehyde, postfixed in OsO<sub>4</sub>, and embedded in SPURR resin (Polysciences). Light microscopy was analyzed with a Leica Light Microscope (Leica) equipped with a Coolscan video camera for computerized images (RS Photometrics) while transmission electron microscopy was performed with the EM 109 Zeiss (Oberkochen).

Immunohistochemical and Immunofluorescence Analysis of GATA-1 Expression. For immunohistochemistry, paraffin embedded samples were cut, stained with the anti-GATA-1 monoclonal antibody (Santa Cruz Biotechnology, Inc.), and developed with the Ultrastain polyclonal HRP Immunostaining kit (Ylem) as described by the manufacturers. Immunofluorescence studies were performed on cytcentrifuged cells fixed in 4% paraformaldehyde for 10 min, washed in PBS, and saturated for 30 min with NET.
cells was also established in collagen–based semisolid cultures (Mega-Cult™-C; StemCell Technologies Inc.) as described by the manufacturer. In brief, 10^5 light density spleen cells or unfracti-
one bone marrow cells were added to the basal medium sup-
plemented with SCF, IL-3, EPO, and TPO (at the same concen-
trations described above) and gel formation was induced by
adding cold collagen solution. The mixture (0.75 ml) was dis-
pensed in duplicate wells of the Mega-Cult™-C chamber slide
and incubated for 7 d. Gels were then dehydrated, acetone fixed,
and stained with toluidine blue.

Culture of BMMC. Marrow and spleen light density cells
(1–2 × 10^6 cells/ml) were cultured for up to 21 d in Iscove’s
modified Dulbecco’s medium supplemented with FBS (10% vol/
vol), 4 mmol/L glutamine, 50 U/ml penicillin, and 50 U/ml
streptomycin sulfate and then stimulated with 100 ng/ml SCF and
10 ng/ml IL-3 as previously described (10, 11).

Infection with Phosphoglycerate Kinase Gene (PGK)-GATA-1 Ret-
oviruses. BMMC were transduced with the retroviral vector
containing the mouse GATA-1 gene under the control of the
human PGK promoter as previously described (42). In brief,
BMMC (10^5 cells) were cocultivated either with the GP plus
E86 producer cell line, which was derived from NIH 3T3 mo-
lecularly engineered to express viral gag/pol and ecotropic env
genes (43), or with NIH 3T3 (2 × 10^5 cells each), as control, in
Iscove’s modified Dulbecco’s medium supplemented with FBS
(10% vol/vol; Hyclone), 4 mmol/L glutamine, 50 U/ml penicil-
lin, 50 μg/ml streptomycin sulfate, 6 μg/ml polybrene (Sigma-
Aldrich), 10 ng/ml SCF, 10 ng/ml IL-3, 1 U/ml EPO, and 50
ng/ml TPO. 48 h later, the nonadherent cells were recovered
from the flasks and transferred in the same culture media fresh-
ly made without polybrene and additionally analyzed 10 and 17 d
later. Because the packaging cell line produces ≈10^6 viral particle
per ml of culture (42), >90% of the GATA-1-transduced cells
incubated for 48 h under these infection conditions reached high levels of
GATA-1 by immunofluorescence.

Serotonin Release Assay. Cells (10^5/ml) were incubated for
6 h at 37°C with 84.0 Ci/nmol[^3]H]serotonin (5-hydroxy-
[^3]H)tryptamine trifluoroacetate, 2 μCi/ml; Amersham Biosci-
ces), washed twice, and incubated again (20 × 10^6 cells/ml) for
1 h on ice either with the monoclonal mouse anti–DNPL-IgE
(10 μg/ml) or with medium. After incubation, cells were washed
again, divided into aliquots (0.4 × 10^6 cells/50 μl), and stimu-
lated for 15 min at 37°C with either medium or 2 μg/ml rat
monoclonal α-lgE (R35-72; BD Biosciences), 1 μg/ml DNPL-
human serum albumin (Sigma-Aldrich), or 1 μg/ml ionomycin
(Sigma-Aldrich). Reactions were terminated with 50 μl cold
Hank’s balanced salt solution. The cells were removed by centrif-
ugation and the level of[^3]H]serotonin in the supernatants was
measured with the Packard 1600TR liquid scintillator counter
(PerkinElmer). The total amount of[^3]H]serotonin incorporated
by the cells was determined by lysing nonstimulated cells with 1%
Triton X-100. The levels of serotonin released upon stimulation
were expressed as absolute counts per minute and as percent of
the total amount of[^3]H]serotonin incorporated (cpm in supernat-
ants ± cpm in cell lystate × 100; reference 5).

RNA Isolation and Semiquantitative RT-PCR Analysis. Total
RNA was prepared with a commercial guanidine thiocyanate/
phenol method (Trizol; GIBCO BRL) using 20 μg glycogen
(Hoffmann LaRoche Ltd.) as a carrier. 1 μg total RNA was re-
verse transcribed at 42°C for 30 min in 20 μl of 10 mM Tris-
HCl, pH 8.3, containing 5 mM MgCl2, 1 U RNase inhibitor,
2.5 U Moloney Murine Leukemia Virus reverse-transcriptase,
and 2.5 μM random hexamers (all from PerkinElmer). Gene-ex-
pression was analyzed by amplifying 2.5 μL reverse-transcribed cDNA in 100 μL of 10 mM Tris-HCl, pH 8.3, containing 2 mM MgCl2, dNTP (200 μM each), 0.1 μCi [α-32P]dCTP (specific activity 3,000 Ci/mmol; Amersham Biosciences), 2 U AmpliTaq DNA polymerase, and sense and 100 nM each antisense primers specific for β2 microglobulin (39), GATA-1 (39), GATA-2 (39), MITF (44), MC-CPA (45), MMCP-6 (45), and MMCP-7 (46). PCR conditions were as follows: 60 s at 95°C, 60 s at 55°C, and 60 s at 72°C for β2 microglobulin, GATA-1, and MMCP-6, and 60 s at 95°C, 60 s at 60°C, and 60 s at 72°C for GATA-2, MITF, and MMCP-7. All of the reactions were performed using a GeneAmp 2400 thermocycler (PerkinElmer) and analyzed in the linear range of amplification (20–35 cycles in all of the cases). Positive (RNA from adult marrow and 32D cells) and negative (mock cDNA) controls were included in each experiment. 20 μL aliquots were removed from the PCR mixture after selected cycles of amplification, and the amplified bands were separated by electrophoresis on 4% polyacrylamide gel for semiquantitative amplification analysis (47). Gels were dried using a Biorad apparatus (Hercules) and exposed to Hyperfilm-MP (Amersham Biosciences) for 2 h at −70°C. All procedures were performed according to standard protocols (48).

Statistical Analysis. Statistical analysis was performed by analysis of variance (Anova test) using Origin 3.5 software for Windows (Microcal Software Inc.).

Results

GATA-1low Mice Contain Many Mast Cell Precursors but Normal Numbers of Morphologically Abnormal Mast Cells in Connective Tissues and Peritoneal Lavage. Histological sections of the ears from the GATA-1low mice and their normal littermates are presented in Figs. 1 and 2. No difference was observed between the number of mast cells revealed by toluidine blue and alcian blue staining in the wild-type animals (Fig. 1). In contrast, in the GATA-1low animals, numerous metachromatic granule-containing cells but normal numbers of mature mast cells were detected by toluidine blue and alcian blue/safranin staining, respectively, in the connective tissue just above the middle cartilage (the trabeculated structure recognizable between two skin layers at the lower level of magnification in Fig. 1 B) of the ear (Figs. 1 and 2; reference 49). The frequency per mm² of total granule-containing cells in the connective region of the ear and skin from the GATA-1low mice and from littersmates was 991 ± 226 versus 259 ± 193 (ear) and 475 ± 14 versus 112 ± 18 (skin), respectively (P < 0.01 in both cases), whereas the frequency of mature mast cells in mutants versus controls was 363 ± 17 versus 193 ± 70 (P < 0.05) in the connective tissues of the ear and 157 ± 37 versus 104 ± 15 (P < 0.08) in the skin (Table I).

Similar to findings in connective tissues, peritoneal lavage from the mutant mice contained more metachromatic granule-containing cells than lavage from controls, whereas they contained similar numbers of alcian blue+ cells (Table I). The frequencies of the two cell populations in peritoneal lavage of the GATA-1low mice and normal littersmates was 11.2 ± 1.4 versus 3.3 ± 0.7 (P < 0.001) and 4.4 ± 0.4 versus 3.1 ± 0.6 (P < 0.05), respectively. Of note, although dividing cells were never detected in lavages from normal mice, dividing cells represented 0.3% of all the granule-containing cells present in lavages of the GATA-1low mice. The higher incidence of immature mast cells in peritoneal lavages of the GATA-1low mice was confirmed by FACS® determination of the frequency of cells expressing c-kit and FcεRI (Fig. 3 A). In lavages of both mutant and normal mice, the majority (>75%) of the cells were B220+ (B cells; not depicted). Lavages from the mutant mice contained more c-kit+ cells than those from normal mice (4–6% vs. 1–2%, respectively), although the frequency (and the levels of expression) of FcεRI+ cells was markedly reduced in the GATA-1low samples (30 vs. 50% of the c-kithigh cells, respectively; Fig. 3 A).

Mast cells of the GATA-1low animals presented an abnormal morphology characterized by a larger size (Figs. 1 and 2). Morphological differences were confirmed by observations under transmission electron microscopy of thin sections from the ears (Fig. 2, E and F). This analysis not only confirmed the larger size of the mutant cells, but revealed further abnormalities that included more dispersive chromatin structure and heterogeneity in size and electron density of cytoplasmic granules (50).

To detail the molecular defects of the GATA-1low mast cells, the levels of expression of the mast cell–specific proteases MMCP-6, MMCP-7, and MC-CPA were analyzed by semiquantitative RT-PCR in unfractionated or purified c-kithigh cells from peritoneal lavages of GATA-1low and normal littermates (Fig. 3 C). In agreement with the low frequency of mast cells in the peritoneal lavages, most of the genes investigated were not amplified from cDNA of unfractionated cell preparations. On the other hand, all of the genes considered were amplified from cDNA from c-kithigh cells purified from normal peritoneal lavage (Fig. 3 C). In contrast, when cDNA from c-kithigh cells purified from GATA-1low animals was used as template, fragments specific for MC-CPA were never amplified and amplification of MMCP-6 was markedly reduced. Interestingly, GATA-2 was amplified at the same levels from c-kithigh cells purified from both mouse strains whereas GATA-1 was amplified with cDNA from wild-type but not GATA-1low littersmates.

To confirm that the GATA-1low mutation impaired GATA-1 expression in mast cells, the presence of GATA-1 protein was compared in ear and peritoneal lavage cells by immunohistochemical (Fig. 2) and immunofluorescence (unpublished data) microscopy, respectively. GATA-1 protein was readily detectable in mast cells of the normal mice with both techniques, but was barely detectable in GATA-1low cells (Fig. 2, C and D).

To clarify why the connective tissues of GATA-1low mice contained normal numbers of mature cells in spite of their high content of mast cell precursors, the frequency of apoptotic cells in the connective tissues was evaluated by TUNEL staining of ear sections (Fig. 4). Very few (<19 nuclei/mm²) fluorescent nuclei were identified in ear sections of normal mice (Fig. 4, A and C). In contrast, high numbers of fluorescent cells (134 ± 2/mm²) were observed in ear sections of GATA-1low mice in which high numbers...
of mast cells had been identified (Fig. 4, B and D). The cells with apoptotic nuclei were positively identified as mast cell precursors by toluidine blue staining (not depicted).

Hematopoietic Tissues from the GATA-1 low Mice Contain High Numbers of a “Unique” Trilineage (E-Mk-Mast Cell) Progenitor Cell. It has been reported that hematopoietic tissues from the GATA-1low mice contain high numbers of E and Mk progenitor cells whereas the number of myeloid progenitors is normal (36–39). It has also been demonstrated that tissues of mutant mice contain a unique progenitor cell, bipotent for the E and Mk lineage, which gives rise to colonies with a typical morphology and whose cells, once transferred into fresh medium, proliferate for at least a couple of passages (36). Similar colonies (the morphology of one of them is presented in Fig. 5 A) were also detected in this study. However, when single colonies were harvested from the dish and individually transferred onto glass slides for morphological analysis, it was recognized that they contained, in addition to E and Mk cells,
granule-containing cells that resembled mast cell precursors (Fig. 5 B, Mc). To exclude the possibility that such a mast cell component represented an artifact due to cell contaminants picked up from the methyl-cellulose, marrow and spleen cells from the GATA-1low mice were also cultured in dishes made semisolid with collagen and stimulated with the same mixture of growth factors used for the standard cultures. After 7 d, the collagen gels were dried and stained with toluidine blue and/or with acetyl-cholinesterase. Also in this analysis, GATA-1low, but not normal marrow and spleen cells, gave rise in 7 d to colonies that were identical in situ as containing erythroblasts, megakaryocytes (acetylcholinesterase*), and mast cells (metachromatic granule-containing cells; unpublished data). This unique trilineage progenitor cell is termed CFU-EMkMc. CFU-EMkMc were below the level of detection in marrow, spleen, and blood cultures of normal littermates (Table II). In contrast, they represented 65–72% of all progenitor cells present in the marrow and spleen of GATA-1low animals (Table II). As many as 315 ± 65 and 800 ± 150 CFU-EMkMc-derived colonies were detected per 10^6 nucleated cells of the spleen and marrow from GATA-1low mice. These colonies were detectable at a frequency of 12 ± 4 per µL in the blood of the GATA-1low mice (Table II).

The cells present in the CFU-EMkMc-derived colonies were further characterized by FACS® analysis. Cells pooled from normal BFU-E-derived colonies were analyzed in parallel as control (Fig. 5 C). The cells were double stained with either TER-119/2D5 to detect E and Mk cells, or with CD117 that recognizes c-kit and CD34 to detect mast cell precursors. The majority of the cells pooled from the BFU-E-derived colonies cultured from normal mice ex-
pressed, as expected, TER-119 and/or 2D5 but very few, if any, expressed high levels of c-kit and/or CD34 (Fig. 5 C). c-kit<sup>high</sup> and CD34<sup>high</sup> cells were also below the level of detection in pools of CFU-GM–derived colonies obtained from the same cultures (unpublished data). In contrast, only some cells from the CFU-EMkMc–derived colonies expressed TER-119 and/or 2D5 whereas 16% of them coexpressed high levels of CD34 and of c-kit (Fig. 5 C). c-kit<sup>high</sup> CD34<sup>high</sup> cells were positively identified as mast cells by sorting and May–Grunwald staining (not depicted).

To further characterize the differentiation potential of mast cells present in the CFU-EMkMc–derived colonies, cells pooled from these colonies (as well as cells pooled from normal BFU-E– and CFU-GM–derived colonies obtained from normal mice) were cultured in liquid culture stimulated with SCF and IL-3, plus or minus a murine fibroblast feeder layer (Fig. 6, NIH 3T3). Cells pooled from normal BFU-E– or CFU-GM–derived colonies failed to prolifer-

### Table I. Frequency of Mast Cell Precursors and Mature Mast Cells in Ear and Skin Sections and in Peritoneal Lavages of Normal and GATA-1<sub>low</sub> Mice

<table>
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<th>Ear (cells/mm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Skin (cells/mm&lt;sup&gt;2&lt;/sup&gt;)</th>
<th>Peritoneal lavages (percent of total nucleated cells)</th>
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<tr>
<td></td>
<td>Toluidine blue&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Alcian blue&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Toluidine blue&lt;sup&gt;+&lt;/sup&gt;</td>
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<tr>
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<td>132</td>
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<tr>
<td>Mean (±SD)</td>
<td>259 ± 75</td>
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<tr>
<td>GATA-1&lt;sub&gt;low&lt;/sub&gt;</td>
<td>1,160</td>
<td>330</td>
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<td>991 ± 226</td>
<td>333 ± 17</td>
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<tr>
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<td>&lt;0.05</td>
<td>&lt;10&lt;sup&gt;−5&lt;/sup&gt;</td>
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The results obtained from three individual GATA-1<sub>low</sub> and normal littermates are presented (each line an individual mouse). <sup>a</sup>0.3% of the cells were in morphologically recognizable mitosis.

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Figure 3. Mast cells in peritoneal lavage from GATA-1<sub>low</sub> mice express lower levels of FceRI and of the proteases MMCP-6 and MC-CPA than the corresponding cells from normal littermates. (A) The FACS<sup>®</sup> analysis of cells from peritoneal lavage from normal and GATA-1<sub>low</sub> mice stained with PE c-kit and FITC-FceRI is presented. Cells from the peritoneal lavage of the mast cells deficient W/W<sup>v</sup> mice (reference 51) were analyzed in parallel as negative control. The gating used for the FceRI analysis is shown in a representative dot plot of PE c-kit versus side scatter analysis. Very similar c-kit/side scatter dot plots were observed in all the cases. The isotype control for the FceRI analysis is not shown because it is superimposable to the histogram obtained with cells from the W/W<sup>v</sup> mouse. (B) The gating used to purify c-kit<sup>high</sup> cells from the peritoneal lavage and a representative reanalysis after sorting of the purified cells is presented. In all of the cases, >80% of the sorted cells were c-kit<sup>high</sup> upon reanalysis. The semiquantitative RT-PCR analysis for the expression of β<sub>2</sub> microglobulin (β2-MG), GATA-1, GATA-2, MMCP-6, MMCP-7, and MC-CPA in unseparated or c-kit<sup>high</sup> cells of peritoneal lavage from GATA-1<sub>low</sub> and normal littermates is presented in C. Each product was amplified for increasing number of cycles (20, 25, 30, and 35), as indicated by the triangle on the top of the panels. Similar results were obtained in two additional experiments.
ate and survived for 1–2 wk or less when transferred into liquid culture (Fig. 6 E). In contrast, cells harvested at day 7 from CFU-EMkMc–derived colonies proliferated for up to 50 d under these conditions (Fig. 6 E). The high proliferative capacity was confirmed in limiting dilution experiments in which single cells were deposited in 96-multiwell plates. Cell proliferation occurred in >98% of the wells provided that the cultures had been stimulated with both SCF and IL-3 (unpublished data).

After the second week of liquid culture, the CFU-EMkMc–derived cells acquired a homogeneous morphology of granule-containing cells that coexpressed high levels of c-kit and CD34 (Fig. 6, F and G) but did not react with alcian blue (unpublished data). Some of the c-kit+ cells coexpressed FcεRI, although the levels of FcεRI expression remained below those expressed by normal BMMC throughout the time in culture (compare Fig. 6 F with 7 A). Of interest, the CFU-EMkMc–derived cells adhered with high frequency (>54% after overnight incubation) to murine fibroblasts (Fig. 6) but expressed an immature phenotype characterized by lack of reactivity with alcian blue (unpublished data) and low levels of FcεRI expression (Fig. 6 G). These results suggest that mast cell precursors within the CFU-EMkMc–derived colonies are unable to complete differentiation.

Differentiation of BMMC Derived from Hemopoietic Tissues of GATA-1low Mice Is Defective In Vitro. Mast cell differentiation has seldom been reported in cultures of normal murine hemopoietic tissues at day 7 (12) but many mast cell–containing colonies were detected at this time in semisolid cultures of tissues from the GATA-1low mice (Figs. 5 and 6). Because CFU-EMkMc represent a unique class of progenitor cells found only in the tissues of the GATA-1low mice, it is possible that the defective mast cells they contain are the result of “abortive differentiation” under nonpermissive culture conditions. To directly compare the maturation of mast cells differentiated in vitro from normal and GATA-1low mice, marrow (and spleen) cells were cultured under conditions specific for the generation of BMMC (10, 12). The results obtained are presented in Fig. 7. Under these conditions, a homogeneous population of BMMC (95% of which were c-kithigh/FcεRIhigh) was observed after 21 d in normal cell cultures. In contrast, marrow cells from the GATA-1low mice yielded a BMMC population, the majority (87%) of which expressed high levels of c-kit, that was composed both by FcεRI+ (16.3% ± 3.7%) and FcεRI+ cells (83.7% ± 3.7%, P < 0.001 with respect to the frequencies observed in cultures obtained from normal mice; Fig. 7 A). In addition, the GATA-1low BMMC expressed less FcεRI on their surface than normal cells (median fluorescence intensity 120 vs. 150 arbitrary units, respectively; Fig. 7 A).

In vitro maturation of GATA-1low mast cells was analyzed further by comparing the levels of [3H]serotonin incorporated and released upon appropriate stimulation with values obtained with normal BMMC (Fig. 7 B). Cells obtained in 21 d of culture from tissues of GATA-1low mice and normal littermates incorporated comparable high levels.
of [3H]serotonin. However, GATA-1low BMMC released significantly less [3H]serotonin after IgE-αlgE stimulation than cells obtained from the normals (36 ± 14 vs. 62 ± 10, respectively; P < 0.01).

In addition, the protease expression profile of GATA-1low and normal BMMC was compared by semiquantitative RT-PCR analysis (Fig. 7 C). Although more MMCP-6 and MMCP-7 fragments were amplified with cDNA from GATA-1low than from wild-type BMMC, MC-CPA fragments were amplified only with cDNA from wild-type cells (Fig. 7 C).

These results indicate that the GATA-1low mast cells mature poorly in vitro even under specific culture conditions.

Forced GATA-1 Expression Restores the Mast Cell Differentiation Potential of GATA-1low BMMC In Vitro. To clarify whether the defective mast cell differentiation observed in cultures from the GATA-1low mice was a direct consequence of the mutation affecting GATA-1 expression, we examined whether reexpression of GATA-1 would restore the in vitro maturation potential of GATA-1low BMMC. GATA-1low cells, obtained at day 7 under BMMC-specific conditions, were cocultured either with NIH 3T3 or with the NIH 3T3-derived cells producing the PGK-GATA-1 virus (42). 48 h later, nonadherent cells were harvested and transferred into fresh medium. At days 10 and 17 after infection, cells were analyzed for their surface antigenic profile and GATA-1 expression. The expression of mast cell-
Table II. Frequency of Progenitor Cells in the Marrow and Spleen of Normal and GATA-1low Animals

<table>
<thead>
<tr>
<th>Number of progenitor cells</th>
<th>Normal mice</th>
<th>GATA-1low mutants</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CFU/10^5 cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFU-E</td>
<td>149 ± 33</td>
<td>165 ± 33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>165 ± 25</td>
<td>145 ± 16</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CFU-EMkMc</td>
<td>0</td>
<td>800 ± 150</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CFU/10^5 cells)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFU-E</td>
<td>15 ± 3</td>
<td>110 ± 21</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>40 ± 5</td>
<td>56 ± 16</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CFU-EMkMc</td>
<td>0</td>
<td>315 ± 65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(CFU/10 μL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFU-E</td>
<td>1 ± 1</td>
<td>14 ± 3</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>3 ± 2</td>
<td>9 ± 2</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>CFU-EMkMc</td>
<td>0</td>
<td>12 ± 4</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Results are presented as the mean (±SD) of at least four independent experiments performed in duplicate for at least eight individual mutants and eight normal littermates analyzed.

Discussion

We have demonstrated that mast cell differentiation is defective in mice harboring a targeted deletion that removes upstream enhancer and promoter elements of the GATA-1 gene. The mutation affects all stages of mast cell differentiation. More specifically, it alters the commitment process by inducing the formation of high numbers of a unique class of progenitor cells (the CFU-EMkMc), induces an increase in proliferation that is coupled by increased cell death (but on balance generating more live cells than dead) of the precursor cells, and finally, impairs the expression of the FcεRI receptor and of the MC-CPA in mature mast cells. Our findings, therefore, implicate GATA-1 as a critical transcription factor for proper mast cell development.

At the level of the progenitor cell compartments, the most striking alteration was reflected by the presence of a unique class of cells, the CFU-EMkMc, which are apparently committed toward the E, Mk, and mast cell lineage (Fig. 5). The CFU-EMkMc gave rise to colonies in only 7 d of culture and their frequency was as high as 800 ± 150 and 315 ± 65 progenitor per 10^5 mononuclear bone marrow and spleen cells, respectively (60–70% of all the progenitor cells present in these organs). Because CFU-EMkMc were not detected from normal mice, their relationship to the pathway of normal hematopoietic differentiation is unclear. In the hierarchy of normal hematopoietic cells, mast cell differentiation potential is thought to be lost before the restriction toward E/Mk differentiation occurs. In fact, mast cells are detected in single cell deposition cultures of purified common myeloid progenitors and not in those of the purified common E/Mk ones (54). It is possible, however, that reduced GATA-1 expression at the progenitor cell level might alter the commitment process itself.
thereby allowing E/MK progenitor cells to retain mast cell differentiation potential (55).

At the precursor level, approximately three times more mast cells were found in the connective region of the skin (Figs. 1 and 2) and in peritoneal lavage (Table I) of the mutant mice. Conversely, granule-containing cells were observed in the connective region but not in the mucosa of the stomach (unpublished data). An increased number of mast cell precursors might result from either increased proliferation capacity and/or a block in terminal maturation. The first possibility is supported by the presence of many (0.3%) dividing mast cells in peritoneal lavage and the high proliferative capacity exhibited by GATA-1low BMMC in vitro (>98% of which were capable to proliferate in single cell cloning experiments). However, cultured mast cells typically have high proliferative capacity (12) and even normal mature cells purified from the tissues of the mice may regain proliferative potential once induced to degranulate (i.e., they are de-differentiated; reference 56). The fact that c-kit^{high} FCεRI^{-} cells were still detectable after 21 d in BMMC-specific cultures seeded with marrow (and spleen) cells of mutant origin (Figs. 6 and 7) favors the possibility that the increased proliferation is a direct result of a block in the cellular maturation induced by the mutation.

In spite of the increased frequency of mast cell precursors in tissues of the GATA-1^low mice, the number of mature alcian blue^+ safranin^+ cells in the same tissues was not significantly higher or only modestly (20–50%) higher than...
normal (Fig. 1). On the other hand, high numbers of apoptotic (TUNEL+; Fig. 4) precursor cells were detected in tissues. It is possible that mutant mast cell precursors, as mutant BMMC in culture, are unable to progress in their maturation process and therefore activate an apoptotic program. Adult GATA-1low mice maintain normal hemoglobin and hematocrit as increased apoptosis observed at the pro-erythroblasts level is counter balanced by high output from the progenitor cell compartment (38, 39). Because in the case of the erythroblasts, apoptosis is a direct consequence of reduced GATA-1 expression (57), we suggest here that GATA-1 is also involved in preventing death of mast cell precursors.

The mature mast cells identified in connective tissue and peritoneal lavage exhibit altered morphology that includes large size, abnormal chromatin organization, and the presence of heterogeneously sized cytoplasmic granules (Fig. 2). In addition, they expressed lower levels of FceRI on their surface and did not express MC-CPA (Fig. 3). These findings are superficially reminiscent of abnormalities in megakaryocyte maturation in the absence of GATA-1. Therefore, we considered whether mast cell maturation is blocked in the GATA-1low mice. In both semisolid cultures and under BMMC-specific culture conditions, cells with the profile of mast cell precursors (c-kithigh, CD34+) were detected as early as day 7 of culture of mutant cells compared with 21 d in cultures of normal cells. Furthermore, maturation of mutant cells was incomplete. Cells with alcian blue+ metachromatic granules were not seen (Figs. 6, 8, and unpublished data). Additionally, GATA-1low BMMC expressed lower levels of FceRI (Figs. 6, F and G, and 7 A), released less [3H]serotonin after IgE-αδγE stimulation (Fig. 6 B) than normal BMMCs and did not express MC-CPA (Fig. 7), which is the only mast cell–specific protease whose expression has been demonstrated to be under GATA-1 control (32).

The similarities between the defects at the level of the mast cell lineage and those observed in E and Mk cells suggest but do not prove that the impaired mast cell differentiation observed in the GATA-1low mice was the direct consequence of a decreased GATA-1 expression in the cells of this lineage. The GATA-1low animals compensate for the defective hemopoiesis induced by the mutation by establishing a complex homeostatic mechanism that includes in-

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**Figure 7.** BMMCs obtained from marrow of GATA-1low mice express lower levels of FceRI and MC-CPA and release less [3H]serotonin after IgE-αδγE stimulation than the normal cells. (A) FACS® analysis for the expression of c-kit and FceRI of BMMC obtained after 21 d of culture under BMMC-specific conditions from normal mice and their GATA-1low littermates are presented on the top and bottom panels, as indicated. Dot plots for side scatter and c-kit expression and histograms for FceRI expression of the c-kit+ gated cells are presented on the left and right, respectively. Negative controls were represented by cells labeled with an irrelevant isotype-matched antibody and for convenience are only presented for the FceRI analysis. The results are representative of those obtained in five separate experiments (each one started with cells harvested from a different animal). (B) Level of serotonin (as cpm/10⁵ cells) released upon IgE-αδγE stimulation by BMMC obtained after 21 d of culture under specific culture conditions from GATA-1low mice (gray bars) and their normal littermates (open bars). Positive and negative controls were represented by cells stimulated with IgE alone, medium plus αδγE, and medium, human serum albumin, DNP, and Ionomycin, as indicated. The levels of total serotonin that had been incorporated by the cells was measured by lysing the BMMC in Triton X-100. The results are presented as the mean (±SD) of at least five separate experiments performed in duplicate. (C) Semi-quantitative RT-PCR analysis for the expression of β2 microglobulin (β2-MG), GATA-1, GATA-2, MITF, MMCP6, MMCP7, and MC-CPA genes in cells obtained after 21 d under BMMC-specific culture conditions. Each product was amplified for an increasing number of cycles (20, 25, 30, and 35), as indicated by the triangle on the top of the panel. Similar results were obtained in two additional experiments.
creased growth factor gene expression (such as TGF-β and platelet-derived growth factor) in the marrow microenvironment (40) and extensive extramedullary hemopoiesis in the spleen (39). It remained possible that the alterations in the mast cell differentiation pathway observed in these animals might be secondary to the mutation itself and the consequence of the alterations observed in the microenvironment. Although homogeneous, and therefore deprived of accessory cells BMMC populations obtained from the mutant mice did not progress properly along the differentiation pathway even when cultured for an extensive period of time (>45 d) under highly specific culture conditions, it could be argued that such a lack of differentiation was the result of an in vivo priming by “nonpermissive” growth factor stimulation. To determine if the abnormal differentiation we observed in vitro was due to insufficient expression of GATA-1, we transduced GATA-1low BMMC with a retrovirus harboring GATA-1 cDNA. After infection, full mast cell differentiation was restored. Thus, the defects we describe in GATA-1low mast cells in vitro result from lower than normal GATA-1 expression. Whether cells entirely lacking GATA-1 would differ in phenotype is under study.

In conclusion, mice with a mutation in the upstream enhancer and promoter of the GATA-1 gene exhibit a complex phenotype that includes impaired mast cell differentiation. From our findings we infer that GATA-1 has a critical role in the proper differentiation of mast cells. In some respects this requirement mimics the role of GATA-1 in both E and Mk. The recent report that targeted deletion of the
high affinity binding site in the GATA-1 promoter leads to selective loss of the eosinophil lineage in vivo (26) and our current data suggest that GATA-1 function is important in the control of inflammation and allergic reactions.

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