Essential and Instructive Roles of GATA Factors in Eosinophil Development

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Abstract

GATA transcription factors are major regulators of hematopoietic and immune system. Among GATA factors, GATA-1, GATA-2, and GATA-3 play crucial roles in the development of erythroid cells, hematopoietic stem, and progenitor cells, and T helper type 2 (Th2) cells, respectively. A high level of GATA-1 and GATA-2 expression has been observed in eosinophils, but their roles in eosinophil development remain uncertain both in vitro and in vivo. Here we show that enforced expression of GATA-1 in human primary myeloid progenitor cells completely switches myeloid cell fate into eosinophils. Expression of GATA-1 exclusively promotes development and terminal maturation of eosinophils. Functional domain analyses revealed that the COOH-terminal finger is essential for this capacity while the other domains are dispensable. Importantly, GATA-1–deficient mice failed to develop eosinophil progenitors in the fetal liver. On the other hand, GATA-2 also showed instructive capacity comparable to GATA-1 in vitro and efficiently compensated for GATA-1 deficiency in terms of eosinophil development in vivo, indicating that proper accumulation of GATA factors is critical for eosinophil development. Taken together, our findings establish essential and instructive roles of GATA factors in eosinophil development. GATA-1 and GATA-2 could be novel molecular targets for therapeutic approaches to allergic inflammation.

Key words: GATA-1 • GATA-2 • eosinophil • myeloid cell • allergy

Introduction

Eosinophils play important roles in host immune defense against helminthic parasites and in the pathophysiology of allergic diseases. Eosinophils develop from progenitor cells in the bone marrow under the control of cytokines including IL-3, GM-CSF, and IL-5 (1). In allergic diseases, eosinophils are specifically recruited to the site of allergic inflammation through specific upregulation of adhesion and chemoattractive molecules as well as increased production of eosinophils in the bone marrow (2).

Transcriptional regulation is a key step in the commitment and differentiation of hematopoietic cells (3, 4). Through detailed analyses of eosinophil-specific promoters, GATA-1, CCAAT/enhancer binding protein (C/EBP)* factors, and RFX have been implicated in the eosinophil-specific gene expression (5–7). Among them, C/EBP and GATA-1 have been characterized in the chicken transformed hematopoietic system, in which C/EBP factors drive eosinophil differentiation of the chicken transformed
hematopoietic progenitor cells (8) and an intermediate level of GATA-1 reprograms chicken myelomonocytic cell lines into eosinophils (9). On the other hand, friend of GATA-1 (FOG-1), a cofactor for GATA-1, inhibits eosinophil differentiation (10). These findings indicate that GATA-1, C/EBP, and FOG-1 act within a regulatory loop during the chicken eosinophil development. Consistent with these findings, C/EBPα-deficient mice shows a selective block in the development of eosinophils as well as neutrophils (11). Furthermore, we have recently shown that enforced expression of C/EBPα in human CD34+ myeloid progenitor cells facilitates both eosinophil and neutrophil development (12). In contrast, the role of GATA-1 in eosinophil development has not yet been characterized in primary hematopoietic cells or in genetically altered mice.

GATA factors comprise a family of transcription factors that have highly conserved zinc finger DNA binding domains. Among six members, GATA-1, GATA-2, and GATA-3 play major roles in hematopoietic and immune system, especially in the development of erythroid cells, hematopoietic stem and progenitor cells, and Th2 cells, respectively (13–17). In addition, expression of GATA-1 and GATA-2 are specifically observed in eosinophils and mast cells during myeloid differentiation (18). We have previously demonstrated that GATA-1 and GATA-2 are differentially expressed during mast cell development and play important roles in establishing diverse mast cell population (19). In this study, we analyzed the roles of GATA-1 and GATA-2 in eosinophil development both in vitro and in vivo and disclosed their novel functions in organizing immune system.

Materials and Methods

Mice. Generation of mice with an erythroid promoter–specific “knock-down” allele of the GATA-1 gene (GATA-1-0.05 mice) has been described previously (14). IE3.9int is the mouse GATA-1 gene regulatory region that contains 3.9 kb of 5′ sequence to the IE exon, the IE exon itself, the first intron, and a part of the second exon of the GATA-1 gene. IE3.9int is capable of conferring the complete GATA-1 expression profile to a reporter transgene in hematopoietic cells (20). Generation of IE3.9int-directed green fluorescent protein (GFP) transgenic mice and rescue experiments of GATA-1-0.05 mice by IE3.9int-directed GATA factor transgenes have been described (20, 21).

Production of Retrovirus. A series of mouse GATA-1 mutant cDNAs were described previously (21). Dominant negative-type GATA genes were generated by replacing NH2-terminal portion of GATA-1 (amino acids 1–167) and GATA-2 (amino acids 1–259) with the engrafted repression domain (amino acids 2–298; reference 22). All cDNAs were FLAG-tagged at the NH2 terminus, then cloned into the retrovirus vector GCsam, which has an LTR derived from MSCV and intact splice donor and splice acceptor sequences for generation of subgenomic mRNA (23). To produce the recombinant retrovirus, the retrovirus vector was transfected into 293gp cells (293 cells containing the gag and pol genes but lacking an envelope gene) along with 10A1 env expression plasmid (pCL-10A1) by CaPO4 coprecipitation and the supernatant from the transfected cells was collected to infect cells.

Preparation of Human Primary and Cultured Cells. Human umbilical cord blood samples were obtained, with informed consent, from placentas of full-term normal newborn infants. After isolation of mononuclear cells by density gradient centrifugation, CD34+ hematopoietic progenitors were obtained using magnetic bead separation (Miltenyi Biotech). In all experiments, ~95% of purified cells were positive for CD34 as judged by specific antibody (23). For reverse transcription (RT)-PCR analysis, cord blood CD34+ cells and glycophorin A+ erythroblasts were purified by cell sorting on a FACS Vantage™ (Becton Dickinson). To obtain eosinophils, cord blood CD34+ cells were incubated in the presence of 50 ng/ml stem cell factor (SCF), IL-3, and GM-CSF for 5 d, then further incubated in the presence of IL-5 (50 ng/ml) only for 10 d. To exclude macrophages, cultured cells were stained with PE-conjugated CD14 (BD Pharmingen), and then CD14-negative cells were collected by cell sorting. May–Grünewald Giemsa staining revealed almost all cells collected are eosinophils.

Transduction of CD34+ Cells. CD34+ cells were prestimulated in IMDM (Sigma-Aldrich) supplemented with 10% FBS, 50 ng/ml SCF, thrombopoietin (TPO; kindly provided by KIRIN, Tokyo, Japan), IL-6, and Flt-3 ligand (PeproTech) for 20 h. After replating onto recombinant fibronectin fragment–coated culture dishes (Takara Shuzo) containing virus supernatant and 5 μg/ml protamine sulfate (Sigma-Aldrich), cells were centrifuged at 1,000 g for 30 min. Transduction was repeated with fresh virus supernatant every 12 h three times. At 60 h after the first transduction, cells were stained with PE-conjugated anti–CD34 (BD Pharmingen). Cells positive for both CD34 and enhanced GFP (EGFP) were selected by cell sorting and subjected to subsequent analyses. At this time point, around 85% of the cells were still positive for CD34 (23).

In Vitro Colony Assay and Liquid Culture. CD34+ cells transduced with indicated retroviruses were plated in methylcellulose medium (StemCell Technologies, Inc.) or cultured in IMDM with 10% FBS. Cytokines were supplemented to culture at the concentrations of 50 ng/ml human SCF, GM-CSF, IL-3, and IL-5 (PeproTech), and 5 U/ml human erythropoietin (kindly provided by Chugai Pharmaceutical Inc., Tokyo, Japan). The culture dishes were incubated at 37°C in a 5% CO2 atmosphere. Colony numbers were counted at day 14. To check the development of IL-5–responsive eosinophils, cells were cultured in the presence of SCF and GM-CSF for the first 5 d. Then, cells were further incubated in the presence of IL-5 alone. Alternatively, preculture media were supplemented with IL-3 in addition to SCF and GM-CSF to facilitate development of eosinophil progenitors.

Analysis of Eosinophil Development in Mice. The fetal liver cell suspensions were prepared by dissecting E11.5 fetal livers by pipetting. The bone marrow cell suspensions were prepared from adult mice and were overlaid with sodium metrizoate solution (1.086 g/ml; Nycomed), then centrifuged at 400 g for 20 min. The low-density cells were harvested and stained with anti-lineage marker antibodies (Mac-1, Gr-1, B220, CD4, CD8, and TER119; BD PharMingen), then lineage marker–negative (Lin−) cells were collected by cell sorting. The fetal liver and Lin− bone marrow cells were plated in methylcellulose medium (StemCell Technologies, Inc.) in the presence of mouse IL-5 (50 ng/ml; Genzyme) or in the presence of mouse SCF, IL-3 (10 ng/ml), and human erythropoietin (5 U/ml). Colony numbers were counted at day 10.

Flow Cytometric Analysis. Expression of the cell surface and cytoplasmic antigens was analyzed on a FACS Vantage™. To detect cell surface antigens, cells were stained with allophycocyanin...
To detect cytoplasmic major basic protein (MBP), cells were fixed in 4% paraformaldehyde and permeabilized in 0.3% saponin, then stained with anti–human MBP (Nichirei) followed by PE-conjugated anti–mouse immunoglobulins (BD PharMingen). Cells that marked with propidium iodide were gated out as dead cells.

**Results**

**Expression of GATA Family Genes in Eosinophils.** High levels of mRNA expression of GATA genes have been reported in human primary eosinophils (18). In this study, RT-PCR analysis also demonstrated that expression of GATA-1 and GATA-2 in human eosinophils is comparable to that in hematopoietic progenitor cells and/or erythroblasts. In contrast, expression of GATA-3 was not significant in eosinophils (Fig. 1 A). Next, we analyzed expression of GATA-1 in mouse eosinophils. We previously generated transgenic mice bearing IE3.9int-directed GFP (20). IE3.9int is the GATA-1 gene regulatory region that contains 3.9 kb of 5’ sequence to the IE exon, the IE exon itself, the first intron, and a part of the second exon of the GATA-1 gene. IE3.9int is capable of conferring the complete GATA-1 expression profile to a reporter transgene in hematopoietic cells (20). IL-5 is a late-acting cytokine that specifically supports eosinophil proliferation. In the presence of IL-5 alone, bone marrow cells from transgenic mice predominantly gave rise to GFP-positive colonies (Fig. 1 B). All the GFP-positive colonies consisted of mature eosinophils, which were positive for fast green staining that detects eosinophil granules (data not shown). On the other hand, the colonies of neutrophils and/or macrophages formed in the presence of IL-3 did not express GFP at all (data not shown). These findings indicate that the transcription of GATA-1 gene is active in mouse eosinophils.

**Role of GATA Factors in Eosinophil Development In Vitro.** Roles of GATA factors in eosinophil development have not yet been characterized in primary hematopoietic system. By using retrovirus, we expressed GATA-1, GATA-2, and their dominant negative-type genes in human CD34+ hematopoietic progenitor cells. The dominant negative GATA-1 and GATA-2 contain engrailed repression domain instead of their own NH2-terminal activation domain (Fig. 2 A), which strongly repress GATA-dependent transcriptional activation. The retroviral vector drives expression of both GATA-related genes and EGFP from a single bicistronic message. After transduction, cells positive for both CD34 and EGFP were selected by cell sorting and subjected to in vitro assay.

To evaluate the effects of GATA-1 and GATA-2 on eosinophil commitment, we first assessed the frequency of eosinophil colony formation. In the presence of SCF and GM-CSF, CD34+ cells transduced with either GATA-1 or GATA-2 gave rise to comparable number of colonies to the control. Surprisingly, in contrast with the control that predominantly gave rise to granulocyte/macrophage colonies, CD34+ cells expressing either GATA-1 or GATA-2 exclusively gave rise to eosinophil colonies (Fig. 2 B). Eosinophil colonies consisted of mature eosinophils, which were positive for fast green staining that detects eosinophil

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**Figure 1.** Expression of GATA family genes in eosinophils. (A) Expression of GATA family genes in human eosinophils. RT-PCR was performed on normalized cDNA templates from cord blood CD34+ cells, and CD34+ CD34+ erythroblasts, and eosinophils obtained by in vitro culture (details in Materials and Methods). Lane H2O represents the negative control without template. (B) Expression of GATA-1 in mouse eosinophils. Eosinophil colonies were generated from bone marrow cells of transgenic mice bearing IE3.9int-directed GFP in the presence of IL-5 alone. Bright field microgram (left) and fluorescence microgram (right).
granules (Fig. 2 C). There was no significant difference in erythroid colony formation between the control and GATA-expressing cells (Fig. 2 B; BFU-E).

In liquid culture supplemented with SCF and GM-CSF that allows myeloid cell development, cells transduced with either GATA-1 or GATA-2 showed slightly decreased cell growth compared with the control, and cells transduced with dominant negative GATA genes showed even lower cell growth rate (Fig. 3 A). To check the development of IL-5–responsive eosinophil progenitors, the transduced cells were cultured in the presence of SCF and GM-CSF for the first 5 d, and then cytokines were replaced with IL-5, the cytokine that specifically supports eosinophil proliferation. Only cells transduced with GATA-1 or GATA-2 proliferated in an IL-5–dependent manner (Fig. 3 A). Next, we supplemented culture medium with IL-3 in addition to SCF and GM-CSF. Under this cytokine condition, eosinophil development is further promoted and even the control cells showed IL-5–dependent cell growth. However, the cells transduced with dominant negative GATA genes did not respond to IL-5 at all (Fig. 3 A), indicating an crucial role of GATA factors for the development of eosinophil progenitors in vitro. Flow cytometric analysis revealed that cells expressing GATA genes failed to develop CD14+ macrophages, but gave rise to MBP-positive eosinophils (Fig. 3 B). Cytological analyses again demonstrated the eosinophilic features of transduced cells, which were positive for both EPO and fast green staining (Fig. 3 C). These data clearly show that GATA-1 and GATA-2 have a strong instructive ability to render myeloid progenitor cells to commit into eosinophils and to support their terminal differentiation.
GATA-1 Functional Domains Responsible for Eosinophil Development. To clarify the requirement for GATA-1 functional domains during eosinophil development, we introduced a series of GATA-1 mutants into CD34<sup>+</sup> progenitor cells. The GATA-1 mutants used are an NH<sub>2</sub>-terminal deletion (ΔNT), an NH<sub>2</sub>-terminal zinc finger deletion (ΔNF), a COOH-terminal zinc finger deletion (ΔCF), and a mutant that lacks both the NH<sub>2</sub> terminus and the NH<sub>2</sub>-terminal zinc finger (ΔNTNF; reference 21). We further generated an additional mutant with a single amino acid substitution of valine 205 into glycine (V205G) that abolishes interaction with FOG-1 (V205G; Fig. 4 A). In the colony assay supplemented with SCF and GM-CSF, CD34<sup>+</sup> progenitor cells transduced with wild-type GATA-1, ΔNF, or V205G gave rise to comparable number of eosinophil colonies (Fig. 4 B). Notably, CD34<sup>+</sup> cells transduced with ΔNT gave rise to a higher number of eosinophil colonies. CD34<sup>+</sup> cells transduced with ΔNTNF formed slightly decreased number of eosinophil colonies. Cells with ΔCF were even more inefficient in forming eosinophil colonies and formed a significant number of granulocyte/macrophage colonies. Importantly, the eosinophil colonies generated from cells transduced with ΔNTNF or ΔCF were much smaller in size than the others (data not shown).

In liquid culture supplemented with SCF and GM-CSF, cells transduced with ΔNT and ΔNTNF showed comparable cell growth with the control while the others showed retarded cell growth (Fig. 4 C, left panel). EPO staining revealed that ΔNT, ΔNF, and V205G efficiently...
drive eosinophil development while ΔNTNF is less efficient and ΔCF barely induces eosinophil development (Fig. 4 D). As expected from these data, IL-5–responsive cell growth was promoted with cells transduced with wild-type GATA-1, ΔNT, ΔNF, or V205G, and was most evident with ΔNT-transduced cells. In contrast, cells with ΔNTNF poorly responded to IL-5 and ΔCF did not respond at all (Fig. 4 C, right panel). All these data indicate that both NT and NF domains are dispensable in promoting eosinophil development, and the CF domain is absolutely required in this process.

**Essential Role of GATA Factors in Eosinophil Development In Vivo.** Analyses of definitive hematopoiesis in the GATA-1–deficient fetal liver thus far have demonstrated defects in differentiation specific to erythroid and megakaryocytic lineages (13–15). Eosinophil development occurs in the fetal liver from the definitive hematopoietic stem cells. To determine the role of GATA-1 in eosinophil development in vivo, we analyzed the development of eosinophil progenitor cells in the GATA-1–deficient fetal livers (Table I). We previously generated mice with an erythroid promoter-specific mutant allele of the GATA-1 gene (GATA-1.05 mice), in which GATA-1 was expressed at ~5% of the wild-type level (14). Because GATA-1 is located on the X chromosome, all male embryos hemizygous for the mutation show GATA-1–deficient phenotypes. As previously observed, the mutant fetal livers showed defective erythropoiesis and contained much less nucleated cells than the wild-type livers. Nonetheless, they contained similar numbers of colony forming units to the wild-type livers. Notably, IL-5–responsive eosinophil colonies were formed from wild-type fetal liver cells but not from mutant fetal liver cells, indicating that eosinophil progenitor cells do not develop in the absence of GATA-1. We next analyzed if transgenic expression of GATA factor genes could rescue deficient eosinophil development in GATA-1.05 mice. We previously succeeded to rescue the embryonic lethal phenotype of GATA-1.05 mice by GATA factor transgenes under the control of the GATA-1 IE3.9int gene (20). Although rescued mice by GATA-2 but not by GATA-1 transgene develop anemia after birth, they survive and were subjected to analysis for eosinophil development. As expected from in vitro data in Figs. 2 and 3, GATA-2 as well as GATA-1 transgenes efficiently compensated for GATA-1 deficiency in terms of eosinophil development in vivo (Table II).

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<th>GM/Mix</th>
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<th>GM/Mix</th>
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E11.5 fetal liver cells were plated in methylcellulose medium supplemented with IL-5 alone (for CFU-Eo) or SCF, IL-3, and erythropoietin (for CFU-GM/Mix). Colony numbers were counted at day 10. CFU/Mix, mixed-CFU. Numbers of nucleated cells recovered from the fetal liver, CFU per liver and CFU per 10⁵ nucleated cells are presented.

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Discussion

Eosinophils develop from myeloid progenitor cells along with neutrophils and macrophages. In this study, we demonstrated that GATA-1 strongly promotes eosinophil commitment of primary myeloid progenitor cells and supports their terminal differentiation. This effect was much more drastic than that of GATA-1 to reprogram chicken transformed myeloblasts into eosinophils. In addition, we clearly showed an essential role of GATA-1 in eosinophil development in vivo. These data establish GATA-1 as an essential regulator for eosinophil development.

Thus far, C/EBPα is known to be essential for eosinophil development (11). However, C/EBPα is also crucial for neutrophil development (11). Enforced expression of C/EBPα in human CD34+ myeloid progenitor cells facilitates both eosinophil and neutrophil development (12). In contrast, GATA-1 exclusively promoted eosinophil development in this study. With respect to induction of eosinophil development, GATA-1 is more specific and efficient than C/EBPα. These data indicate that the expression of GATA-1 in combination with C/EBPα could be the critical determinant for eosinophil lineage commitment. On the other hand, GATA-2 showed instructive capacity comparable to GATA-1 in vitro and efficiently compensated for GATA-1 deficiency in terms of eosinophil development in vivo, indicating that proper accumulation of GATA factors is essential for eosinophil development. However, endogenous GATA-2 did not compensate for the loss of GATA-1 function in vivo as has been observed in erythropoiesis (13, 14). These data may also indicate functional distinctions between GATA-1 and GATA-2.

Table II. Transgenic Rescue of GATA-1.05 Mutant Mice

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<th>Genotype</th>
<th>CFU per 10⁵ Lin⁻ cells</th>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
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<td>GATA-2 transgene</td>
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Lineage marker–negative (Lin⁻) bone marrow cells that did not react with anti-lineage marker antibodies (Mac-1, Gr-1, B220, CD4, CD8, and TER119) were collected by cell sorting and plated in methylcellulose medium supplemented with IL-5 alone (for CFU-Eo) or SCF, IL-3, and erythropoietin (for CFU-GM/Mix). Colony numbers were counted at day 10. Numbers of CFU per 10⁵ Lin⁻ cells are presented.

The role of GATA-2 in eosinophil development in vivo remains to be determined.

Structural analysis of GATA-1 in eosinophil development revealed that the NT and NF domains are dispensable in promoting eosinophil development. In addition, interaction with FOG-1 was not vital. These profiles of domain requirement are in good agreement with those of GATA-1 in the chicken eosinophil development that does not require the NF domain (10), but contrast with those of GATA-3 in Th2 development, in which each of the functional domains is required (24). Although we cannot directly compare these data with those of in vivo rescue experiments in GATA-1.05 mutants, we previously showed that either NT or NF domain is dispensable during primitive but not definitive erythropoiesis (21). As CF encompasses the DNA-binding domain, these data indicate that the site-specific DNA binding is sufficient for the initiation of eosinophil development from primary myeloid progenitor cells that retain multipotency in differentiation. Interestingly, ∆NT was most efficient in promoting eosinophil development, indicating that the NT domain mediates some negative regulations for eosinophil commitment or proliferation. The NT domain could play distinct functions in eosinophils from those in other cell lineages. Although ∆CF do not bind to DNA, it slightly promoted eosinophil development. This effect could be mediated by indirect mechanism, for example, by sequestrating GATA-interacting factors such as FOG-1, which could negatively regulate GATA-1 in eosinophils (10), thereby enhancing endogenous GATA-1 function.

To inhibit Th2 lymphocyte–mediated allergic inflammation, many approaches have been taken that target GATA-3, such as local delivery of GATA-3 antisense oligonucleotides (25). Our data clearly implicated GATA-1 and GATA-2 in the regulation of eosinophil development and function. In addition, we have previously demonstrated that GATA-1 and GATA-2 play differential but important roles in mast cell differentiation and function (19). Because eosinophils and mast cells are largely responsible for development and progression of allergic inflammation, and GATA-1 and GATA-2 are predominantly expressed in eosinophils and mast cells in the site of allergic inflammation, GATA-1 and GATA-2 could be new therapeutic targets for the local treatment of allergy.

We thank Dr. Y. Shiina (Shiina Hospital, Ibaraki, Japan) and Dr. N. Sonia (University of Minnesota) for providing us with human cord blood and 293gp cells, respectively, Dr. Y. Yamaguchi (Center for Sleep Respiratory Disorder, Fukuoka, Japan) for valuable discussion, Y. Morita for technical assistance, and M. Itoh for excellent secretarial assistance.

This work was supported in part by grants from the Ministry of Education, Culture, Sport, Science and Technology, and Core Research for Evolutional Science and Technology (CREST) of Japan Science and Technology Corporation.

Submitted: 1 February 2002
Revised: 12 April 2002
Accepted: 15 April 2002
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