Coordinated regulation of transcription factors through Notch2 is an important mediator of mast cell fate


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Mast cells are thought to participate in a wide variety of pathophysiologic conditions. Mechanisms of regulation, however, of mast cell production and maturation are still to be elucidated. Mast cell developmental process is likely to be profoundly affected by cell-autonomous transcriptional regulators such as the GATA family and CCAAT/enhancer binding protein (C/EBP) family members. Extracellular regulators such as stem cell factor and IL-3 have essential roles in basal and inducible mast cell generation, respectively. The relationship, however, between the extracellular signaling and cellular transcriptional control is unclear, and the trigger of the mast cell development remains elusive. Notch signaling plays a fundamental role in the lymphopoietic compartment, but its role in myeloid differentiation is less clear. Here, we demonstrate that Notch signaling connects environmental cues and transcriptional control for mast cell fate decision. Delta1, an established Notch ligand, instructs bone marrow common myeloid progenitors and granulocyte-macrophage progenitors toward mast cell lineage at the expense of other granulocyte-macrophage lineages, depending on the function of the Notch2 gene. Notch2 signaling results in the up-regulation of Hes-1 and GATA3, whereas simultaneous overexpression of these transcription factors remarkably biases the progenitor fate toward the mast cell-containing colony-forming cells. C/EBPα mRNA was down-regulated in myeloid progenitors as a consequence of Hes-1 overexpression, in agreement with the recent proposal that the down-regulation of C/EBPα is necessary for mast cell fate determination. Taken together, signaling through Notch2 determines the fate of myeloid progenitors toward mast cell-producing progenitors, via coordinately up-regulating Hes-1 and GATA3.

CCAAT/enhancer binding protein α | GATA3 | Hes-1 | Delta1 | myeloid progenitor

Mast cells are thought to participate in a wide variety of physiologic and pathologic processes. In addition to their involvement in allergic disorders and protective immune responses to parasites, mast cells are important in a broader sense in both innate and acquired immunity (1, 2). Mast cells migrate from the bone marrow at an immature differentiation stage and complete the maturation process in the peripheral tissues under the influence of local growth and differentiation factors (2). These environmental cues tailor the mast cell phenotype to carry out functions specific to each peripheral tissue. The main factors influencing mast cell number and phenotype include stem cell factor (SCF), which is a ligand for c-Kit, IL-3, and T helper type II (Th2) -associated cytokines, such as IL-4 and IL-9 (2). Signal transduction through the phosphatidylinositol 3-kinase pathway, Ras-mitogen-activated protein kinase pathway, Janus kinase-signal transducer and activators of transcription (JNK-STAT) pathway (3), etc., in the presence of IL-3 and SCF, might coordinate the lineage-specific transcription factors, but there is a disconnect between our understanding of cytokine signaling and transcription factor regulation. The generation of mast cells has been shown to depend on cooperative interplay between regulatory proteins: PU.1 up-regulation, GATA2 up-regulation (4), and CCAAT/enhancer binding protein α (C/EBPα) down-regulation (5).

Notch receptor-mediated signaling has a fundamental role in cell fate determination in a variety of animals. In the mammalian immune system, Notch signaling is involved in the commitment and differentiation of T cells, development of splenic marginal zone B cells, and differentiation and functional modulation of mature T cells (6, 7). Questions remain, however, about whether and how Notch signaling regulates immune cells other than T and B cells. To the best of our knowledge, there are only two reports (8, 9) describing the relationship between Notch signaling and mast cells. According to those papers, Jagged-1, a Notch ligand (8), and Notch2 (9), are highly expressed in mast cells.

Here, we demonstrate that Notch signaling has a significant role in mast cell development. Using Notch2–null bone marrow cells, we show that Notch2 signaling promotes cell fate determination of bone marrow progenitors toward the progenitors having mast cell differentiation capacity at the expense of those that lose it. Furthermore, we demonstrate that the coordinated regulation of two transcription factors, Hes-1 and GATA3, determines mast cell fate under Notch signaling. Overexpression of Hes-1 in myeloid progenitors represses C/EBPα mRNA expression, in agreement with the proposal that the C/EBPα down-regulation provides essential condition for mast cell development (5).

These findings suggest that Notch signaling, a key regulator of lymphocytes, more broadly affects cells of the immune system through dynamic regulation of transcription factors.

Results

Notch2-Mediated Signaling Facilitates Mast Cell Lineage Development at the Expense of Granulocyte/Macrophage Development from Both Common Myeloid Progenitors (CMPs) and Granulocyte-Macrophage Progenitors (GMPs) in Vitro. We cultured isolated CMPs and GMPs in SCF, IL-3, IL-6, and thrombopoietin (TPO) for 7 days with

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See Commentary on page 7629.

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plate-fixed Delta1-Fc chimeric protein, a soluble Notch ligand. In the culture using control Fc protein, instead of Delta1-Fc, the vast majority of the cells were Gr1+Mac1+ granulocytes/macrophages. Compared with this, Lin- c-Kit+ FcεRI+ mast cells were highly enriched, whereas there were fewer Gr1+ Mac1+ granulocytes/macrophages with Delta1-Fc (Fig. 1A). The cell number was not significantly different in the culture with Delta1-Fc from the culture with the control Fc protein on day 7, when the flowcytometric analysis was performed (Fig. 1B). As shown, time courses of cell growth were different between the cultures with Delta1-Fc and control, because of the difference in the differentiation fate. Morphological observation confirmed the conversion from granulocytes/macrophages with the control Fc to mast cells, which contained toluidine blue-positive granules, with Delta1-Fc (Fig. 1C). The expression level of mast cell-specific protease, mast cell protease-5, was higher in Delta1-Fc, whereas the results obtained with littermate Notch2\textsuperscript{lox/lox} mice treated with plpC were virtually the same as those obtained with WT mice.

**Hes-1 Is Up-Regulated Downstream of Notch Signaling but is Insufficient for Mast Cell Derivation.** Quantitative real-time PCR analysis revealed that Delta1-Fc treatment of CMPs or GMPs up-regulated mRNA for Hes-1, a basic helix-loop-helix transcriptional repressor that often functions downstream of Notch signaling (11), within as little as 8 h (Fig. 2A). To investigate whether Hes-1 is a downstream effector of Notch signaling for mast cell enrichment by Delta1-Fc, we retrovirally expressed Hes-1 in CMPs and GMPs. Expression of the Hes-1 protein was confirmed by a Western blot analysis of the retrovirally infected NIH 3T3 cells (Fig. 2B). Derivation of the Lin- c-Kit+ FcεRI+ mast cells, however, was not increased by Hes-1 expression after 8 d of culture (Fig. 2C), whereas there was a relative increase in Lin- c-Kit+ FcεRI+ cells. This observation indicated that the effect of Hes-1 expression on mast cell enrichment was not equivalent to that of Delta1-Fc stimulation, suggesting the presence of an effector molecule other than Hes-1 at the downstream of Notch.
C/EBPα is a critical transcription factor for myeloid differentiation (16), and its down-regulation cooperates with the up-regulation of a GATA transcription factor to instruct mast cell development (5). At 48 h after the initiation of Hes-1 retroviral transduction, C/EBPα mRNA was down-regulated in both CMPs and GMPs (Fig. 4A). The C/EBPα mRNA level was also markedly suppressed in a mouse myeloid cell line 32D that stably expressed exogenous Hes-1 (Fig. 4B). These findings suggest that the C/EBPα down-regulation is the major outcome of Hes-1 up-regulation induced by Delta1–Notch2 signaling in the myeloid progenitors.

Discussion
In the present study, we demonstrate that Notch2-mediated signaling has a significant role in the mast cell derivation from myeloid progenitors such as CMPs and GMPs. This biological effect is probably mediated through coordinated up-regulation of Hes-1 and GATA3 through Notch2 signaling: Hes-1 up-regulation further results in C/EBPα down-regulation, which is important for the blockade of myeloid lineage differentiation. It is not unexpected that Hes-1 up-regulation plays a role in the execution of Notch2 signaling, given the fact that Hes-1 is an established target of Notch signaling in a number of cell systems (17). However, we show in this article that Hes-1 up-regulation...
GATA3, but not GATA2, is a mediator downstream of Notch2 for mast cell developmental decision, together with Hes-1. (A) CMPs and GMPs were stimulated with plate-fixed Delta1-Fc in the presence of SCF, IL-3, IL-6, and TPO for 8 or 48 h. Cells were harvested, and quantitative real-time PCR was performed. GATA2 mRNA levels were slightly decreased rather than increased by Delta1-Fc in GMPs at 8 h, and not significantly different in CMPs treated with Delta1-Fc and control Fc at 8 h or in CMPs and GMPs treated with Delta1-Fc and control Fc at 48 h. (B) Quantitative real-time PCR analyses in CMPs and GMPs revealed that GATA3 mRNA was up-regulated by Delta1-Fc at 48 h, but not at 8 h. Data are presented as mean ± SD; n = 4, P = 0.6857 (CMPs, 8 h); n = 4, P = 0.0571 (GMPs, 8 h); n = 4, P = 0.0286 (both CMPs and GMPs, 48 h) (nonparametric test). (C) CMPs and GMPs were retrovirally transduced with Hes-1-GCDNASam/IRES-NGFR and GATA3-pMys/IRES-GFP and subjected to FACs analysis 8 days after the infection. The c-Kit+/FceRI+ fraction was remarkably enriched in Hes-1 and GATA3 double-positive fraction compared with the Hes-1 or GATA3 single-positive fraction or the mock virus-introduced fraction. (D) The proportions of c-Kit+/FceRI+ fraction are depicted. Each diamond represents a data point. The bars represent ± 2 SD. CMP, n = 6; GMP, n = 4. (E) NGFR and GFP double-positive cells were sorted at 48 h after infection. Two hundred and fifty NGFR and GFP double-positive CMPs and 1,500 GMPs were plated per dish and cultured for 7 days in methylcellulose, supplemented with SCF, IL-3, IL-6, and TPO. Hes-1- and GATA3-coexpressing cells formed mainly mast cell colonies, whereas mock virus-transduced cells formed various colonies including granulocyte, macrophage, or a mixture of these cells. G, granulocyte colonies; M, macrophage colonies; mix, granulocyte/macrophage or mixed colonies; *, colonies mainly consisting of mast cells. The result of a representative experiment is shown (n = 2). (F) Wright-Giemsa staining of Hes-1 and GATA3 coexpressing colony-forming cells. (Original magnification: ×400.) (G) Toluidine blue staining of Hes-1 and GATA3 coexpressing colony-forming cells. (Original magnification: ×400.)

Fig. 3.

represents only a part of signaling downstream of Notch activation. Exogenous Hes-1 expression in CMPs and GMPs resulted in the increase of Lin−c-Kit−FceRI+ cells, obviously different from the Lin−c-Kit−FceRI+ mast cell enrichment that was seen by the Delta1 stimulation. Although the identity of the Lin−c-Kit−FceRI− cells has yet to be determined, these cells may contain immature myeloid progenitors that could differentiate into mast cells if GATA3 would coexist, while granulocytes/macrophages would if other critical molecules (such as C/EBPα) would coexist.

We identified GATA3, not GATA2, up-regulation that complements Hes-1 up-regulation and creates a part of Notch signaling. Involvement of GATA2 is proposed to be important for inhibiting granulocytic differentiation downstream of Notch signaling in both the 32D cell line (13) and mouse hematopoietic progenitor cells (12). Furthermore, GATA2 is required for in vitro mast cell generation (4, 14), and enforced expression of GATA2 instructs the C/EBPα-deficient myeloid progenitors and common lymphoid progenitors to become mast cells in vitro (5). Our conclusion might appear to be inconsistent with those of other articles. We confirmed that the enforced GATA2 and Hes-1 coexpression in CMPs and GMPs resulted in predominant mast cell generation in a manner indistinguishable from that of GATA3 and Hes-1 coexpression (data not shown). This finding indicates that GATA2 and GATA3 have redundant properties when they are expressed exogenously. The result of colony formation assay from CMPs and GMPs with enforced GATA3 and Hes-1 expression indicates that the mast cell derivation is based on the cell fate alteration made in the individual progenitor cells. Because the biological readout for Notch ligand stimulation was virtually the same as that for the GATA3 and Hes-1 coexpression, the substantial mast cell generation at day 7 with Delta1-Fc is likely to be caused mainly by biased cell fate decision in the myeloid progenitors.
Schematic model of Notch signaling in the mast cell system. Shown to be among the mechanisms of Notch-Hes-1-mediated mast cell-mediated intestinal nematode eradication. The pathways the IL-3 gene, whereas IL-3 is the most potent mast cell developed from Notch2-null bone marrow cells as efficiently as WT bone marrow cells were not depleted in naïve status in N2-MxcKO mice (M.S.-Y. Sakata-Yanagimoto et al. 2008). It was recently suggested that down-regulation of C/EBPα expression is a result of environmental regulation of transcription factors is a result of environmental.

In a different line, the introduction of GATA3 alone to thymocytes was recently reported to result in mast cell generation (15). Although this report might appear to be inconsistent with our data, the difference in the starting cell populations could cause the different results. We conclude that the Hes-1 expression, and probably subsequent C/EBPα down-regulation, is required, although not sufficient, for mast cell generation from C/MPS and G/MPS. In contrast, C/EBPα is already down-regulated in thymocytes at the DN1 and DN2 stages (18), and thus, introduction of GATA3 might be sufficient for mast cell generation from early thymocytes. As for the relationship between GATA3 and Notch signaling, both Notch1 and Notch2 are important for the generation of Th2 cells and act by directly inducing transcription of GATA3 and IL-4 (19, 20). It is, however, unclear whether such a direct regulation is applicable to cells in other lineages. In our observation, GATA3 was up-regulated by Delta1-Fc at 48 h but not at 8 h in C/MPS and G/MPS, making it obscure whether GATA3 is a direct target of Notch signaling in these cells. The role of IL-4 in the regulation of GATA3 in these cells remains to be determined.

We and others previously reported that enforced expression of Hes-1 in a 32D cell line inhibits granulocytic differentiation induced by granulocyte colony-stimulating factor (13, 21). In the present study, we demonstrated that C/EBPα down-regulation occurs downstream of Hes-1 in both the 32D cell line and fresh C/MPS and G/MPS. C/EBPα repression by Hes-1 was previously shown to be among the mechanisms of Notch-Hes-1-mediated inhibition of adipogenesis from a preadipocyte cell line (22). Although less remarkable compared with Hes-1 overexpression, Delta1-Fc stimulation also induced C/EBPα repression, in a time course after Hes-1 up-regulation. This finding suggests that the physiologic Hes-1 up-regulation is sufficient for C/EBPα repression (data not shown). Our data, thus, support a paradigm that the Notch-Hes-1/C/EBPα axis consists of a common pathway for differentiation inhibition in a variety of cell lineages. It was recently suggested that down-regulation of C/EBPα followed by up-regulation of a GATA factor orchestrates mast cell differentiation from myeloid progenitors (5). This could be true, but importantly, we demonstrated that such a balanced regulation of transcription factors is a result of environmental signaling through Notch2, rather than a cell-autonomous operation (Fig. 4C).

The physiological significance of Notch2-mediated cell fate bias toward mast cell lineage remains to be determined, because mast cells were not depleted in naïve status in N2-MxcKO mice (M.S.-Y. and S.C., unpublished data). Cultured mast cells were also generated from Notch2-null bone marrow cells as efficiently as WT bone marrow cells. Notably, mast cells are not depleted in mice lacking the IL-3 gene, whereas IL-3 is the most potent mast cell development factor in vitro. However, IL-3-deficient mice are defective in mast cell-mediated intestinal nematode eradication. The pathways and mechanisms responsible for regulating mast cell progenitor recruitment and trafficking are likely to be dynamic and susceptible to modification during inflammation (1). Similarly, Notch2 is required for the proper response of mast cells during nematode infection (M.S.-Y. and S.C., unpublished observation). Notch2-mediated mast cell derivation might also be required for such pathological settings, whereas it is unnecessary for the steady-state mast cell generation.

Materials and Methods

Mice. Notch2floxed mice have been described (10). Mx-Cre transgenic mice (23) were crossed with Notch2flox mice, and the progeny were injected with pIpC (Sigma) seven times every other day from 3 d after birth (25 μg body weight) or three times between 4 and 6 wk of age (20 μg body weight). All experiments were done in accordance with institutional guidelines.

Myeloid Progenitors. Bone marrow cells from each mouse strain studied were incubated with biotinylated antibodies for lineage markers including anti-CD3, anti-CD4, anti-CD8, anti-Gr-1, anti-Ter119, and anti-Gr-1 antibodies (BD Pharmingen) followed by incubation with streptavidin Micro Beads (Miltenyi Biotec). The lineage-marker-negative fraction was separated with an autoMACS separator (Miltenyi Biotec) and incubated with anti-CD34-FITC, anti-CD16/32 (FcγRIII receptor)–phycoerythrin (PE), anti-c-Kit–allophycocyanin (APC), streptavidin peridinin chlorophyll protein PerCP (BD Pharmingen), and anti-Scal-PE/Cy7 (e Bioscience). Lin–c-Kit–Scal–FcγRI–CD34– cells (CMPs and GMPs, respectively) (24) were sorted by a FACS Aria cell sorter (Becton Dickinson).

Ligand Fixation. Delta1-Fc has been described (25). A 24-well nontissue culture plate (Corning, Inc.) was coated with 10 μg/ml of rabbit anti-human IgG (DAKO), blocked with 20% FBS containing Iscove’s medium 1640 (Sigma-Aldrich), and washed with PBS. The Delta1-Fc (3.5 μg/ml) or Fc portion of human IgG (2 μg/ml, Fc protein; ART or Jackson ImmunoResearch Laboratories) was then incubated for 30 min, and supernatants were removed.

Ligand Stimulation of Myeloid Progenitors. Sorted CMPs or GMPs were cultured in Delta1-Fc or control Fc protein-fixed plates in 20% FBS containing Iseove’s modified Dulbecco’s medium (Sigma-Aldrich), supplemented with 50 ng/ml SCF, 20 ng/ml IL-3 (Peprotech), 20 ng/ml IL-6, and 20 ng/ml TPO (gifts from Kirin Pharma Tokyo). On day 7, the cells were incubated with purified isotype IgE (BD Pharmingen) after blocking the Fcγ receptor with anti-CD16/32 (FcγRII/III receptor) antibody, stained with anti-IgE-FITC, anti-Gr-1-PE, anti-Mac1-PE, and anti-c-Kit–APC (BD Pharmingen), and then analyzed by FACS Calibur (Becton Dickinson). Cells cultured for 7 days were also characterized by Wright-Giemsa staining or toluidine blue staining (pH 0.5) on cytospin slides. In some experiments, mRNA was prepared at the indicated time points and quantified by real-time PCR as described below.

Retroviral Transduction. Hes-1 cDNA, a gift from R. Kageyama (Kyoto University, Kyoto, Japan), was subcloned into a retrovirus vector, GCDNas/IM internal ribosome entry site (IRES)-NGFR, a gift from H. Nakauchi (University of Tokyo) and M. Onodera (National Center for Child Health and Development, Tokyo). Cells were transduced with Hes-1 and maintained in 5 ng/ml IL-3 were examined for C/EBPα expression. C/EBPα mRNA was reduced in Hes-1-transduced clones compared with mock-transduced clones. Data were confirmed by experiments using two independent clones. Data are presented as mean ± SD. (C) Schematic model of Notch signaling in the mast cell system.
medium was concentrated, placed in a 24-well nontissue culture dish for 4 h, and precoated with 40 µg/ml of RetroNectin (Takara Bio) overnight at 4°C. C MPs or GMPs were then plated for infection in the presence of 20% FBS, 50 ng/ml SCF, 20 ng/ml IL-3, 20 ng/ml IL-6, and 20 ng/ml TPO. Green fluorescent proteins and/or human NGFR-positive fractions were subjected to FACS analysis 8 days after infection. Otherwise, the infected cells were sorted at 48 h from the initiation of infection with a FACS Aria (Becton-Dickinson) cell sorter and used for colony assay using Methocult M3231 ( Stem Cell Technologies), supplemented with cytokines as described above. Hes-1 stably expressing 32D cells were also infected with the same viral supernatants by using polybrene supplemented with cytokines as described above. Hes-1 stably expressing 32D cells were also infected with the same viral supernatants by using polybrene (Sigma-Aldrich).

RNA Quantitation. Total cellular RNA was extracted with RNeasy (Qiagen) and converted to cDNA with SuperScript III (Invitrogen). GATA2, GATA3, CEBPa, and mouse mast cell protease-5 were analyzed with TaqMan Gene Expression assays (Applied Biosystems). Hes-1 mRNA was measured as described (10). Real-time PCR was performed by using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). All of the data were standardized with 18s ribosomal RNA.

Western Blot Analysis. Virus-infected NIH 3T3 cells were solubilized in lysis buffer containing 1% Triton X-100. The Hes-1 and GATA3 proteins were detected by anti-Hes-1 antibody (H-140; Santa Cruz) and anti-GATA3 antibody (HG3–31), respectively.

Statistical Analysis. Results from two or three independent experiments (n = 2) of quantitative real-time PCR were analyzed by the Mann–Whitney test.

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Fig. S1. The expression levels of Notch1, Notch2, and Notch3 in KSL, CMPs, and GMPs were compared with those in the splenic T and B cells. The expression levels of Notch1 in CMPs and GMPs were lower than that in splenic T and higher than that in B cells. The Notch2 expression levels in CMPs and GMPs were higher than that in splenic T cells and lower than that in splenic B cells. The expression levels of Notch3 in CMPs and GMPs were lower than those in splenic B and T cells. Notch1, Notch2, and Notch3 expression levels were consistently decreased as the cells differentiate from KSL to CMPs and to GMPs.