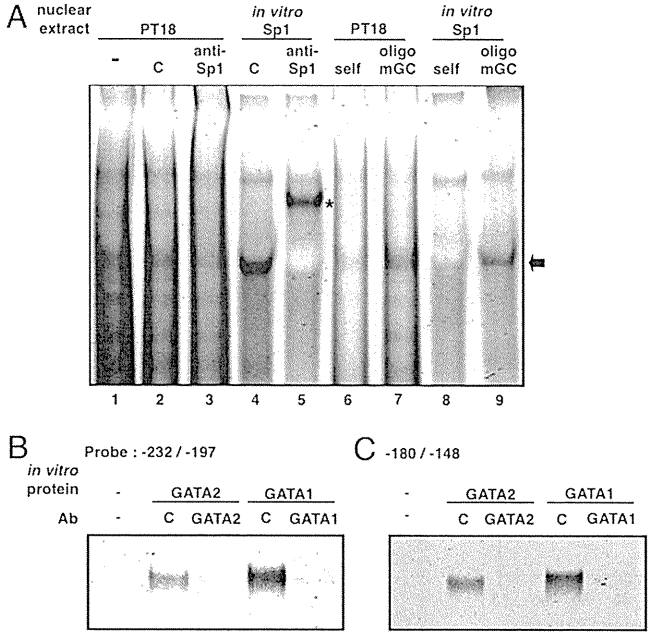

FIGURE 3. Sp1 and GATA1 and/or GATA2 are recruited to the −107/−77 region, and −232/−197 and −180/−148 region, respectively. *A*, EMSAs were performed with the use of 5 pmol FITC-labeled probe, 5 μg of PT18 cell nuclear extract, and in vitro translated Sp1. The arrow indicates the position of the specific band containing probe and Sp1. *Lanes 1–3, 6,* and *7*, Labeled probe and PT18 nuclear extract; *lanes 4, 5, 8* and *9*, labeled probe and in vitro translated Sp1. Additives: *lane 1*, none (−); *lane 2*, isotype control IgG (C); *lane 3*, anti-Sp1 Ab; *lane 4*, isotype control IgG (C); *lane 5*, anti-Sp1 Ab; *lanes 6* and *8*, nonlabeled probe (self); *lanes 7* and *9*, nonlabeled oligonucleotides containing mutated GC-box sequences introduced by nucleotide replacement (oligo mGC). Asterisk indicates the position of the supershifted band containing probe, in vitro translated Sp1 and anti-Sp1 Ab. *B*, GATA1 and GATA2 protein, prepared by an in vitro transcription/translation system, were incubated with 5 pmol of −232/−197 or −180/−148 FITC labeled probes and anti-GATA1 Ab, anti-GATA2 Ab, or isotype control IgG (C).

expression impairs the promoter activity of c-*kit*. Cotransfection of GATA2 siRNA and Sp1 siRNA into BMMCs, which reduced the mRNA levels of GATA2 and Sp1 to the same degree as mentioned above for single siRNA usage, downregulated the mRNA of c-*kit* by ~50% (Fig. 6*C*). To confirm whether the recruitment of GATA2 and/or Sp1 to the c-*kit* promoter region was reduced in siRNA-transfected BMMCs, a ChIP assay was performed after 20 h cultivation following siRNA transfection. As shown in Fig. 6*D*, binding of GATA2 to the c-*kit* promoter was not detected in BMMCs transfected with GATA2 siRNA (siGATA2) (siGATA2/BMMC), and the amount of GATA2 binding to the c-*kit* promoter in Sp1 siRNA-transfected BMMCs (siSp1/BMMC) was ~20% of that in nonsilencing control siRNA-transfected BMMCs (siNega/BMMC). The binding of Sp1 to the c-*kit* promoter disappeared in siSp1/BMMCs, whereas the amount of Sp1 detected in siGATA2/BMMC was almost the same level as that of siNega/BMMC (Fig. 6*F*). The promoter-specific binding of the transcription factors was confirmed by the *cis*-control ChIP assay (Fig. 6*E*, 6*G*). These findings suggest that the recruitment of Sp1 to the promoter region of c-*kit* is necessary for the binding of GATA2.

Repression of GATA2 expression by shRNA resulted in a decrease in the surface expression of c-kit *in PT18*

To further investigate the necessity of GATA2 for c-*kit* expression, GATA2 expression was knocked down in the mouse mast cell line PT18 by introduction of GATA2 shRNA/pSuper (shGATA2/PT18). PT18 cells were used for this experiment instead of BMMCs because 10 d culture of BMMCs under GATA2 knockdown condition (resulting in suppression of c-*kit* expression) to obtain shGATA2-transfectants may affect the viability and/or survival of BMMCs, which are depend on c-Kit ligand SCF, whereas PT18 are growing in SCF-independent manner. After 10 d of culture in growth medium containing puromycin to select transfectants, cells were harvested, and analyzed by flow cytometry. Surface c-Kit expression of shGATA2/PT18 was approximately one tenth lower than that obtained by transfection of empty vector (mock/PT18) (Fig. 7*A*). Densitometry analysis of Western blotting demonstrated that the amount of GATA2 protein in shGATA2/PT18 was reduced to ~20% of that in mock/PT18 (Fig. 7*B*), whereas the protein levels of the ubiquitously expressed transcription factor YY-1 was comparable between both transfectants. The ChIP assay showed that the recruitment of GATA2 to the c-*kit* promoter (−168/+22) was reduced in shGATA2/PT18 (Fig. 7*C*). A significant difference was not detected between anti-GATA2 and isotype control IgG in the *cis*-control region of shGATA2/PT18 and mock/PT18 (Fig. 7*D*). These results suggest that GATA2 is essential for the cell-surface expression of c-Kit in mast cells.

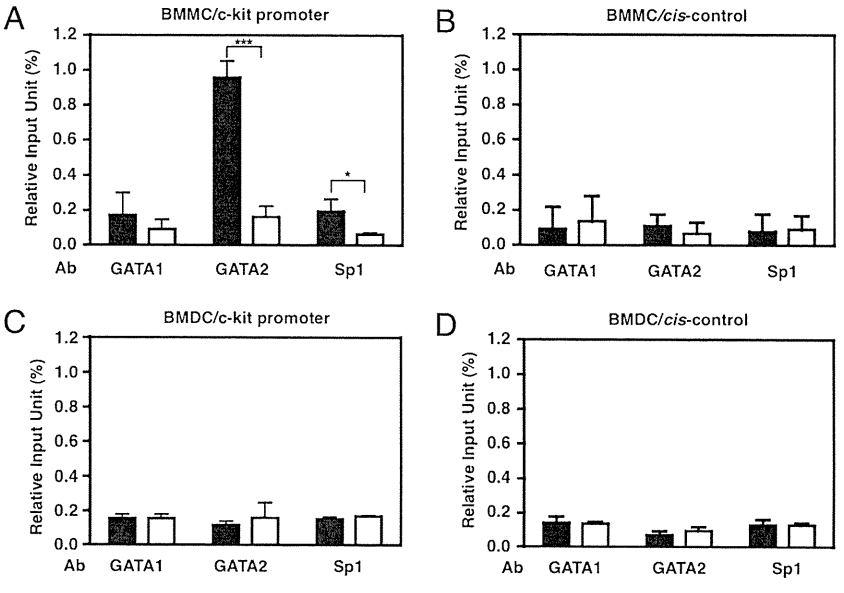

FIGURE 4. GATA2 and Sp1 are recruited to the c-*kit* promoter in mast cells. In BMMCs (*A*, *B*) and BMDCs (*C*, *D*), quantitative analysis of Sp1 and GATA binding to the c-*kit* gene by the ChIP assay using real-time PCR was performed. The closed box indicates anti-GATA1, anti-GATA2, or anti-Sp1 Abs and the open box indicates isotype control IgG. *A* and *C* show the results of PCR with a c-*kit* specific probe, and (*B*) and (*D*) show that with a *cis*-control probe. The results express the mean ± SEM for two PCR with duplicate samples in each of three independent ChIP assays. Relative input units are calculated from Ct values as described in the ChIP assay method.

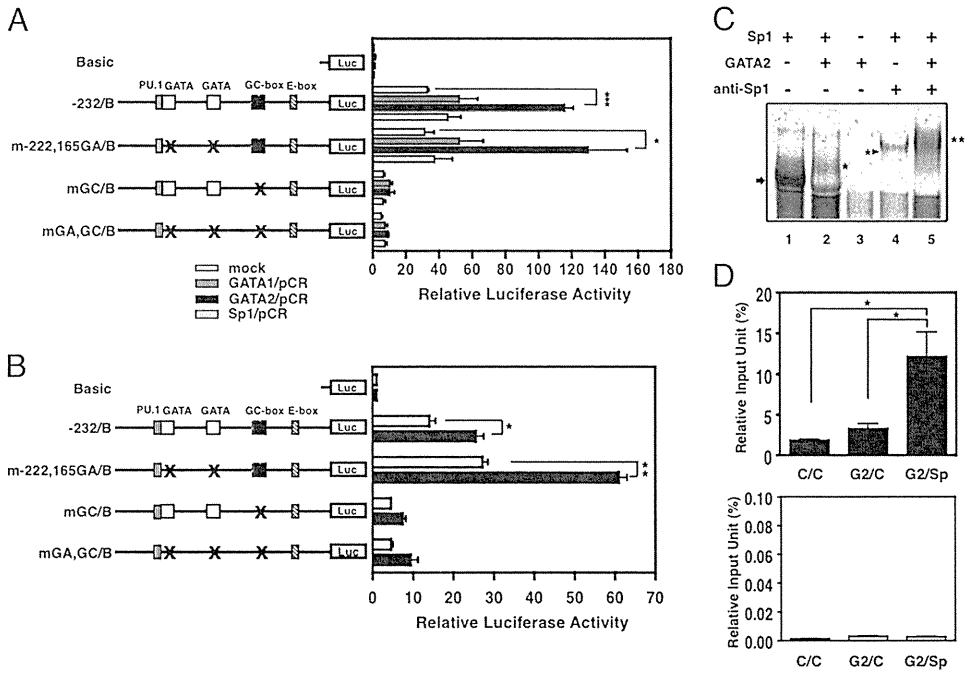

FIGURE 5. The luciferase activity of the c-*kit* promoter was enhanced by GATA2 coexpression in CV1 cells. *A*, A 1 μg amount of each reporter plasmid and 100 ng of expression plasmid were introduced into CV1 cells using FuGene 6. The relative luciferase activity is represented as the ratio to the activity driven by pGL3-Basic (Basic) and pCR3 empty vector (mock). *B*, BMMCs were transfected 5 μg each of reporter plasmids and 3 μg of expression plasmid (pCR3 empty plasmid, open box, or GATA2/pCR, closed box) by electroporation. The ratio of luciferase activity of each construct to that of Basic in the presence of empty expression plasmid was represented as relative luciferase activity. *C*, EMSAs were performed with the use of 5 pmol FITC-labeled probe (−108/−77) and in vitro translated Sp1 and/or GATA2. The arrow indicated the position of the band containing Sp1 and the probe, and the asterisk indicated that of the band containing Sp1, GATA2, and the probe. *Lane 1, 2, 4,* and *5*, In vitro translated Sp1; *lane 2, 3,* and *5,* in vitro translated GATA2; *lane 4* and *5*, anti-Sp1 Ab. Double asterisks indicated the position of supershifted band containing in vitro translated Sp1, GATA2, the probe, and anti-Sp1 Ab. *D*, Re-ChIP analysis. C, isotype control IgG; G2, anti-GATA2 Ab; Sp, anti-Sp1 Ab. The result of PCR using a c-*kit* specific probe (*top*) or a *cis*-contol probe (*bottom*), are shown. The results are expressed as the mean ± SEM for two PCR reactions, with duplicate samples, in each of three independent re-ChIP assays. Relative input units are calculated from the Ct values as described in the ChIP assay method.

Discussion

Although the molecular bases for the transcriptional regulation of c-*kit* in hematopoietic stem cells have been investigated, the mechanisms of c-*kit* transcription in mast cells, which continuously express c-*kit* at a high level, have been unclear. Thus, we investigated c-*kit* transcriptional regulation in mast cells using a reporter assay, EMSA, and a ChIP assay with an RNA interface. In this study, we demonstrated that the transcription factors GATA2 and Sp1 maintain c-*kit* expression cooperatively.

In the reporter assay using a series of 5′- deletion promoter constructs, the mouse c-*kit* promoter was functional only in BMMCs, but not in BMDCs, as shown in Fig. 1, indicating that cell type-specific promoter activity is assigned within the −232/−62 promoter region. Motif analysis using the program TFSEACH (http://www.cbrc.jp/reseach/db/TFSEACH.html) revealed the presence of one PU.1-binding motif, two GATA-binding motifs, one GC-box, and one E-box in the −232/+22 region. These motifs were candidates for *cis*-enhancing elements for the following reasons. GATA and SCL, which binds to the E-box, are predominantly expressed in hematopoietic cells, and the pentameric complex, containing GATA, SCL, E2A, LMO2, and Ldb-1, bind to DNA via the GATA or SCL/E2A or Sp1 motif, either alone or in combination (32, 33). GATA and PU.1 are essential for the generation of mast cells (34). Our reporter assay using various mutated constructs revealed that the GC-box is the essential element for functioning of the c-*kit* promoter (Fig. 2). By EMSA using anti-Sp1 Ab and competitive oligonucleotides, Sp1 was identified to bind the GC-box in the −108/−77 region (Fig. 3*A*). In addition, GATA1 and/or GATA2 possess the ability to bind to two GATA motifs in −232/B by EMSA (Fig. 3*B*). In the ChIP assay, we detected significant binding of Sp1 and GATA2 to the −232/−62 region of the c-*kit* promoter, but GATA1 binding was not observed in BMMCs (Fig. 4*A*). These findings suggest that both Sp1 and GATA2 are recruited to the c-*kit* promoter region and activate the promoter in BMMCs in a cell type-specific manner (Fig. 4*C*). GATA has been shown to bind (A/T)GATA(A/G) or GATC sequences directly (35), and other studies have shown that the activity of GATA protein is modulated by interaction with other factors as follows. In erythrocytes, the complex of GATA1 and cofactor FOG1 coordinates cell maturation, and GATA1 represses activation of erythroid-specific gene expression by direct binding of NuRD/MeCP1 complex to FOG1 (36, 37). In megakaryocytes, the interaction of GATA1 and the SCL complex, which includes SCL, E2A, Ldb1, LMO2, and ETO2 regulate cell maturation (38). In mast cells, recruitment of FOG1 toward GATA1 represses the expression of the FcεRI β-chain, which allows mast cell-specific expression of the β-chain (20). In the current study, the E-box did not contribute to c-*kit* promoter function, suggesting that SCL is possibly not involved in c-*kit* promoter functions. In addition, we did not detect significant recruitment of FOG1 toward the c-*kit* promoter in the ChIP assay (data not shown). Previously, Tsujimura et al. (10) showed that MITF transactivates the c-*kit* promoter via binding to the CACCTG motif at −356/−351. However, in the current study, deletion of −622/−233 significantly increased c-*kit* promoter activity (Fig. 1). Although we do not have evidence to explain this discrepancy, one possible reason for the different results may be that different host cells were used for the reporter assay. In the previous studies, the cell lines, FMA3 and FDC-P1 were used for the reporter assay without exogenous MITF, and

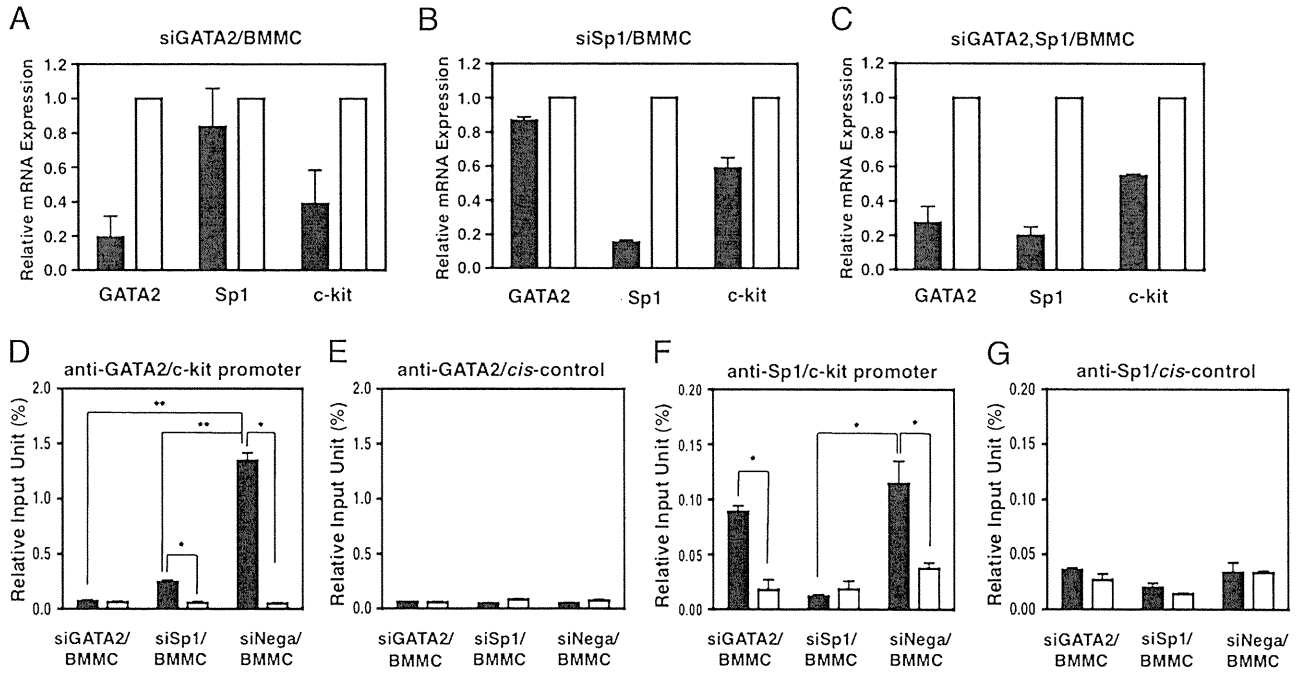

FIGURE 6. Transfection of GATA2 and/or Sp1 siRNA induced c-*kit* mRNA repression in BMMCs. *A–C*, Quantification of GATA2, Sp1, and c-*kit* mRNA in siRNA transfectants, siGATA2/BMMC (*A*), siSp1/BMMC (*B*), and siGATA2, Sp1/BMMC (*C*), using real-time PCR. The relative mRNA expression levels of siRNA transfectants (closed box) are represented as the ratio to that of control siRNA transfectants (open box). *D–G*, siRNA transfectants (siGATA2/BMMC, siSp1/BMMC, and siNega/BMMC) were analyzed with anti-GATA2 Ab (*D*, *E*) or anti-Sp1 Ab (*F*, *G*) in a ChIP assay. The amount of c-*kit* specific promoter (*D*, *F*) or *cis*-control (*E*, *G*) in each immunoprecipitate was quantified by real-time PCR. The closed box indicates the relative input unit by anti-GATA2 or anti-Sp1 Abs and the open box indicates that by isotype control IgG.

NIH/3T3 was used for the MITF-coexpression reporter assay (10), whereas BMMCs were used in the present reporter assay with deletion construct (Fig. 1). Considering that CACCTG functions as a *cis*-enhancing element in a mastcytoma, but not in another myeloid cell line, the effect of MITF may be differentially controlled in a cell type-specific manner. Furthermore, the function of MITF in BMMCs at various developmental stages may be different from its function in mast cell line. Alternatively, it is possible that an element in some other region of −622/−233 might affect the function of MITF. Such an element would be lost in the −622/−233 deletion mutant in the current study but would be retained in the point mutation of CACCTG in the previous study. In our preliminary experiments, MITF was associated with the complex containing GATA2 and chromosome in the re-ChIP assay (data not shown). Further detailed analysis will be required to clarify the complicated roles of each transcription in the function of the c-*kit* promoter.

To identify the mechanisms of the enhancement of c-*kit* promoter activity by GATA2 and Sp1, we performed a reporter assay using various reporter constructs, introduced nucleotide replacements in the GATA and/or Sp1 binding motifs, and GATA2 or Sp1 expression plasmids, and EMSA using in vitro translated Sp1 and GATA2 (Fig. 5). In both of CV1 cells and BMMCs, the reporter activities of the wild-type c-*kit* promoter (−232/B) were enhanced by cotransfection with GATA2 expression plasmid. Although the reporter activity of −232/B and *m*-222,165GA/B were not enhanced by coexpression of Sp1 in CV1 cells, this unexpected result may

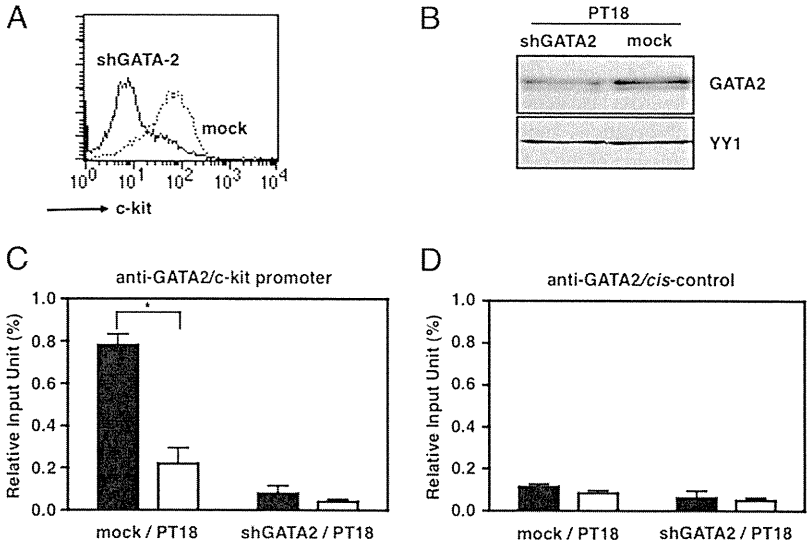

FIGURE 7. Expression of shGATA2 induced the downregulation of c-*kit* transcription and surface expression in PT18. *A*, Surface expression of c-Kit on shGATA2 transfectants. PT18 cells transfected with shGATA2/pSuper-retro-puro (shGATA2, solid line) or empty vector (mock, dotted line) were stained with PE-conjugated anti–c-Kit Ab. *B*, Western blot analysis of shGATA2-transfected PT18 cells. Cells were transfected with shGATA2 or empty vector and cultured in the presence of puromycin for at least 10 d. Cell lysates (1×10^6 cells per lane) were analyzed using anti-GATA2 or anti-YY1 Ab. *C* and *D*, Quantitative analysis of GATA2 binding to the c-*kit* promoter in a ChIP assay using real-time PCR. Chromatin of transfected PT18 cells was immunoprecipitated by anti-GATA2 Ab or isotype control IgG. Precipitated DNA molecules were analyzed by real-time PCR to test for the presence of the c-*kit* promoter (*C*) or the *cis*-control locus (*D*). The open box indicates isotype control IgG and the closed box indicates anti-GATA2 Ab.

have been due to the endogenous expression of Sp1 in CV1 cells. Regardless, coexpression analysis with CV1 indicated that GATA2 transactivates the c-*kit* promoter via the GC-box, independent of the SCL complex (33), because the hematopoietic specific factors, SCL and LMO2, are not expressed in CV1 cells. By EMSA using in vitro translated Sp1 and/or GATA2, GATA2 is suggested to the GC-box in the presence of Sp1, whereas GATA2 alone cannot bind the GC-box. Although the band containing Sp1 and GATA2 was not detected when EMSA was performed using PT18 nuclear extract and the same probe in Fig. 3*A*, this may have been due to dissociation of Sp1 and GATA2 during nuclear extraction, because the re-ChIP assay suggests that Sp1 and GATA2 are simultaneously recruited to the GC-box site in the c-*kit* promoter.

Mutant promoters lacking GATA motifs exhibited higher transcriptional activity compared with that of wild-type in mast cells (Fig. 2). One of the hypotheses of this enhancement may be that mutation of GATA-motif in the promoter, which plays a competitive role against the GC-box in the recruitment of GATA2, increased the amount of GATA2 binding to Sp1 on the GC-box. Alternatively, these GATA-motifs may function as silencing elements that suppress the c-*kit* promoter in other GATA1/2 positive hematopoietic lineage, including erythroids and megakaryocytes. Further analysis of GATA1/2 profiles on these GATA-motifs in various cell-types may provide interesting information regarding the mechanism of cell type-specific c-*kit* expression.

By knockdown of GATA2 and/or Sp1 in BMMCs, c-*kit* mRNA expression was reduced to ~50% (Fig. 6*A–C*), and GATA2 recruitment to the promoter region was reduced by the impaired recruitment of Sp1 in the ChIP assay (Fig. 6*D–G*). Furthermore, c-Kit surface expression of PT18 was reduced by GATA2 knockdown, accompanied with decreased recruitment of GATA2 to the promoter region (Fig. 7). These results indicate that the recruitment of GATA2 and Sp1 to the GC-box mainly enhances the c-*kit* promoter. The c-*kit* mutant promoters lacking GATA motifs was transactivated by GATA2 in the current study, suggesting that GATA2 is involved in c-*kit* promoter function without binding to the GATA motifs, although these GATA motifs possess GATA protein binding affinity. One of the possible mechanisms is indirect interaction with the GC-box via the formation of a complex with Sp1. The ChIP assay with siRNA-transfected BMMCs showed that the recruitment of GATA2 to the c-*kit* promoter is dependent on the presence of Sp1. In addition, the re-ChIP assay showed that both Sp1 and GATA2 are specifically recruited to the region of the GC-box. These data suggest that GATA2 binds to the GC-box via Sp1 binding, and that formation of a complex accelerates the binding of Sp1 to the GC-box. Sp1 physically interacts with GATA1, 2, and 3 and functions as a scaffold for GATA1 binding to the globin gene,- which lacks GATA motifs (39). In addition, GATA3 regulates the dopamine β-hydroxylase gene through physical interaction with Sp1 (40). These observations support our hypothesis that GATA2 regulates the expression of the c-*kit* gene by interaction with Sp1. In any case, our data indicate that GATA2 interacts with the c-*kit* promoter region via Sp1 and maintains expression of the c-*kit* gene in a mast-cell-specific manner, although further analysis is required to clarify the involvement of other transcription factors.

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Disclosures

The authors have no financial conflicts of interest.

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Leptin Enhances Survival and Induces Migration, Degranulation, and Cytokine Synthesis of Human Basophils

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Basophils are the rarest leukocytes in human blood, but they are now recognized as one of the most important immunomodulatory as well as effector cells in allergic inflammation. Leptin, a member of the IL-6 cytokine family, has metabolic effects as an adipokine, and it is also known to participate in the pathogenesis of inflammatory reactions. Because there is an epidemiologic relationship between obesity and allergy, we examined whether basophil functions are modified by leptin. We found that human basophils express leptin receptor (LepR) at both the mRNA and surface protein levels, which were upregulated by IL-33. Leptin exerted strong effects on multiple basophil functions. It induced a strong migratory response in human basophils, similar in potency to that of basophil-active chemokines. Also, leptin enhanced survival of human basophils, although its potency was less than that of IL-3. Additionally, CD63, a basophil activation marker expressed on the cell surface, was upregulated by leptin, an effect that was neutralized by blocking of LepR. Assessments of basophil degranulation and cytokine synthesis found that leptin showed a strong priming effect on human basophil degranulation in response to FcεRI aggregation and induced Th2, but not Th1, cytokine production by the cells. In summary, the present findings indicate that leptin may be a key molecule mediating the effects of adipocytes on inflammatory cells such as basophils by binding to LepR and activating the cellular functions, presumably exacerbating allergic inflammation. *The Journal of Immunology*, **2011, 186: 5254–5260.**

Basophils are the least common circulating leukocytes in human blood. They possess FcεRI, a high-affinity receptor for IgE, abundantly on their surface and, following cross-linkage of IgE on their surface by specific Ags, they degranulate and release chemical mediators such as histamine from their cytoplasmic granules. These cells have long been thought to behave as effector cells in allergic inflammation, since influx of basophils is observed in the upper and lower airways hours after Ag challenge (1, 2). However, new evidence obtained from murine studies clearly revealed that the role of basophils is not restricted to local effector actions, but extends to regulation and modulation of allergic inflammation. These newly uncovered aspects of basophils include initiation of Th2 responses after Ag challenge (3) and regulation of immunological memory (4). As a result, basophils are now increasingly recognized as important immunomodulatory cells in allergic diseases that are attracted to, and activated at, allergic inflammatory sites (5).

We and others have previously reported that several cytokines regulate basophil functions. IL-3, IL-5, and GM-CSF are well-known basophil activators (6–8), and we very recently demonstrated that IL-33 potently activates human basophils directly and also primes them (9). In addition to these cytokines, chemical substances such as fMLP and lipid mediators also possess the ability to modify and regulate basophil functions (10). A better understanding of the precise mechanisms of basophil activation is important for clarifying the pathogenesis of allergic diseases, and it will also potentially lead to the development of new and better therapeutic strategies because basophils are one of the key cell types that potently regulate allergic inflammation.

Today, the incidences of allergic diseases as well as obesity are rapidly increasing, especially in industrialized countries. Obesity is closely related to asthma and airway hyperresponsiveness (11, 12), and the relative risk of asthma rises with increasing obesity. Several clinical observations suggest that obesity worsens asthma control and that asthma in obese subjects has distinct features compared with the disease in nonobese subjects. These observations are that obese asthmatics tend to have a more severe form of asthma (13), respond less well to standard asthma therapy (14), and show resistance to glucocorticoid therapy (15). In fact, weight reduction in obese patients with asthma results in improvement in the asthma severity and symptoms (16). Also, in an animal model, airway responsiveness was shown to be increased in genetically obese mice (17). Based on these observations, obesity and asthma are thought to have a close relationship and there is an urgent need to clarify the precise mechanisms that account for the mutual interaction between these disorders.

In obese subjects, adipocytes secrete increased amounts of adipokines, a series of mediators showing significant metabolic effects. It was recently suggested that some adipokines may also be

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Abbreviations used in this article: Ct, threshold cycle; LepR, leptin receptor; MESF, molecules of equivalent soluble fluorochrome units; RQ, relative quantitation.

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involved in allergic inflammation through modification of immunological responses (18). These adipokines include leptin, a 16-kDa hormone synthesized mainly by adipocytes. Leptin was originally identified as the gene defect responsible for the obese phenotype in ob/ob mice (19), and it is a member of the IL-6 family of cytokines, which have strong structural similarities with G-CSF (20). Leptin receptor (LepR) is a member of the class I cytokine receptor family and, due to alternative splicing, it has six isoforms (LepRa through LepRf). They differ in the length of their intracellular domain, and the long full-length isoform (LepRb) is the receptor responsible for most of the known effects of leptin. These leptin receptors are expressed in various organs, including the lung, kidney, adrenal gland, hematopoietic cells, and bone marrow (21). Also, cells such as neutrophils, monocytes, T cells, and eosinophils have been shown to express functional leptin receptors (22–24), but it has been unclear whether basophils also possess leptin receptors.

On the basis of this background, we conducted analyses to elucidate the effects of this obesity-related mediator, leptin, on human basophil activation. In this study, we report for the first time, to our knowledge, that human basophils express receptors for leptin, and that leptin potently affects basophil migration, survival, CD63 expression, degranulation, and Th2 cytokine synthesis.

Materials and Methods

Reagents

The following reagents were purchased as indicated: human recombinant leptin, human eotaxin/CCL11, and RANTES/CCL5 (R&D Systems, Minneapolis, MN); human MCP-1/CCL2 and IL-3 (PeproTech, Rocky Hill, NJ); human IL-33 (Adipogen, Incheon, South Korea); Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden); PBS and RPMI 1640 medium (Life Technologies, Grand Island, NY); and PIPES (Sigma-Aldrich, St. Louis, MO).

The following Abs were purchased as indicated: mouse anti-leptin mAb (IgG1, clone 44802), mouse IgG1 with irrelevant specificity, normal goat IgG, CFS-conjugated mouse anti-leptin receptor mAb (IgG2b, clone 52263), and CFS-conjugated mouse IgG2b (R&D Systems); goat anti-LepR (Santa Cruz Biotechnology, Santa Cruz, CA); PE-conjugated mouse anti-CD63 (IgG1, clone H5C6) (BD Biosciences, San Jose, CA); PE-conjugated mouse IgG1 and PE-conjugated anti-CD11b mAb (mouse IgG1, clone Bear1) (Coulter Immunotech, Marseille, France); and goat anti-human IgE Ab and FITC-conjugated goat anti-human IgE Ab (Biosource International, Camarillo, CA). Mouse anti-human FcεRI α-chain mAb CRA-1 was provided by Dr. Chisei Ra of Nihon University.

Cell preparation

Leukocytes were isolated from venous blood obtained from consenting volunteers. PBMCs were obtained by the standard density gradient technique (25).

Basophils were semipurified by density centrifugation using Percoll solutions of two different densities (1.080 and 1.070 g/ml), as previously described (9). The purity of these Percoll-separated basophil preparations was usually 5–15%, and the yield was ~0.5–1.0 × 10^5 basophils/ml of peripheral blood. For some experiments, Percoll-separated basophils were further purified by negative selection with MACS beads (basophil isolation kit; Miltenyi Biotec, Belgisch-Gladbach, Germany) according to the manufacturer's instructions (purity, 97–100%).

Eosinophils were purified by density gradient centrifugation followed by negative selection using anti–CD16-bound beads as previously described (25) (purity, 97–100%).

Human neutrophils were separated by density gradient centrifugation followed by negative selection using anti–CD14-bound micromagnetic beads (Miltenyi Biotec) (purity, 97–99%).

Real-time quantitative PCR analysis for LepR

Real-time quantitative PCR analysis was performed using a LightCycler Fast Start DNA Master SYBR Green I kit and LightCycler Fast Start DNA Master HybProbe (Roche Diagnostics, Mannheim, Germany). In brief, total RNA was extracted from highly purified cell preparations from separate donors using an RNeasy Mini kit (Qiagen, Hilden, Germany). Real-time PCR was performed using a LightCycler (Roche Diagnostics). The primers, probes, and standards were designed by Nihon Gene Research Laboratory (Sendai, Japan). Data were calculated as the ratios of the quantities of measured mRNA to β-actin mRNA.

Flow cytometric analysis of surface-expressed molecules

LepR expression in highly purified basophils was analyzed by flow cytometry. MACS-separated basophils were incubated with CFS-conjugated mouse anti-LepR mAb or control CFS-conjugated mouse IgG2b and then analyzed with a FACSCalibur (BD Biosciences, Franklin Lakes, NJ). For some experiments, highly purified basophils were incubated with stimulants in RPMI 1640 medium supplemented with 10% FCS, and after 24 h, cells were stained and analyzed by flow cytometry.

CD63 and CD11b expression levels were analyzed using semipurified basophils. Following stimulation in PIPES buffer containing 25 mM PIPES, 119 mM NaCl, 5 mM KCl, 2 mM Ca^{2+}, 0.5 mM Mg^{2+}, and 0.03% human serum albumin for 60 min, basophils were incubated with either PE-conjugated anti-CD63 mAb, PE-conjugated anti-CD11b, or PE-conjugated control mouse IgG1 and then stained with FITC-conjugated anti-human IgE Ab at 10 μg/ml. Cells showing strong positive staining for IgE were considered to be basophils and were further analyzed for their PE fluorescence. The median values of fluorescence intensity for the basophils were converted to the numbers of molecules of equivalent soluble fluorochrome units (MESF), as previously described (26). Surface expression levels were semiquantified using the following formula: ΔMESF = (MESF of cells stained with specific mAb) − (MESF of cells stained with control IgG).

Chemotaxis assay

Basophil chemotaxis experiments were performed using Chemotaxicell (Kurabo, Osaka, Japan) as previously described (26). Migration was expressed as a percentage of the inoculated cells.

Survival assay

Highly purified basophils were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich). Differential analysis of apoptotic and live cells was performed using a MEBCYTO apoptosis kit (MBL, Nagoya, Japan) and flow cytometry as previously described (27).

Degranulation of human basophils

Basophil degranulation was examined by measuring the supernatant histamine content using semipurified basophils, as previously described (28).

Quantification of basophil-derived cytokines

Highly purified basophils at a cell density of 1 × 10^6 cells/ml were cultured with cytokines in RPMI 1640 medium containing 10% FCS overnight at 37°C, and the supernatant was stored at −80°C until assay. Also, the cell fractions were lysed in ISOGEN (Nippon Gene, Tokyo, Japan), and the total RNA was extracted according to the manufacturer's instructions. Human IL-4 was measured using the Quantikine HS human IL-4 immunoassay (R&D Systems), human IL-13 was measured using an IL-13 ELISA kit (Thermo Fisher Scientific, Rockford, IL), and human leptin was measured using a human leptin immunoassay kit (Invitrogen, Carlsbad, CA). The mRNA expression was analyzed using an Applied Biosystems 7500 real-time PCR system (PE Applied Biosystems, Foster City, CA). The primers and probes for human β-actin, IL-4, IL-13, and leptin were designed by PE Applied Biosystems. For each sample, the differences in threshold cycles (Cts) between the cytokine and β-actin genes (ΔCt_{sample}, $\Delta Ct_{control}$) were determined, and a calibrated ΔCt value (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{control}$) was calculated. Then, the relative quantitation (RQ) values were calculated using the following equation: $RQ = 2^{-\Delta\Delta Ct}$.

Statistics

All data are expressed as the mean ± SEM. Differences between values were analyzed by a Student *t* test.

Results

Human basophils express LepR

In the first series of experiments, we studied the expression of mRNA for the leptin receptor, LepR, in highly purified human basophils, neutrophils, eosinophils, and PBMCs by quantifying the

expression levels of transcripts for LepR by real-time PCR (Fig. 1*A*). Basophils expressed LepR mRNA at a level similar to other granulocytes but lower than the level expressed by PBMCs. We also examined basophil surface expression of LepR by flow cytometry. As shown in Fig. 1*B*, LepR was expressed on the surface of basophils. We next stimulated basophils with potent stimuli, that is, IL-3, IgE aggregation, and IL-33 (9), to see whether they affected the expression level of LepR. Interestingly, basophils stimulated with IL-33 at 100 ng/ml expressed higher levels of LepR, as shown in Fig. 1*C*, whereas basophils stimulated with IL-3 at 300 pM or CRA-1 mAb at 100 ng/ml showed no change in the LepR expression level (data not shown). These results indicate that basophils express LepR at both the mRNA and surface protein levels, and that these expression levels are affected by IL-33. In the following experiments using leptin, we assessed whether LepR expressed on basophils was functional.

Leptin induces migration of human basophils

It has been demonstrated that in vivo administration of leptin attracts inflammatory cells to the sites of allergic inflammation (29). Basophils potentially play a role in allergic inflammation, and for that reason we investigated whether leptin regulates human basophil migration. We found that leptin added to the lower chambers of Chemotaxicell induced dose-dependent migration of human basophils (Fig. 2*A*), showing the strongest effect at a concentration of 3 μM. It is noteworthy that the maximal effect of leptin on human basophil migration was as strong as the effects of several well-known chemokines, including eotaxin, MCP-1, and RANTES. Also, when added together with cells to the upper chambers of Chemotaxicell, leptin at 1–10 μM enhanced basophil migration toward eotaxin (10 nM) (Fig. 2*B*). We found that leptin added simultaneously to both the upper and lower chambers of Chemotaxicell showed a migratory effect on human basophils that was comparable to the effect of leptin added only to the lower chambers, indicating that the effect of leptin on human basophils is both chemotactic and chemokinetic (Fig. 2*B*).

Leptin enhanced survival of human basophils

Leptin has already been shown to enhance the longevity of eosinophil and other inflammatory cells (23, 24, 30, 31). We next examined the effect of leptin on basophil survival. Highly purified basophils cultured for 96 h were analyzed to determine the percentages of live cells and apoptotic cells. As shown in Fig. 3*A*, leptin showed dose-dependent enhancement of basophil survival. The maximal effect of leptin on human basophil survival was seen at a concentration of 10 μM, but it was slightly weaker than the effect of IL-3, the most potent basophil survival factor. Also, the number of apoptotic basophils decreased with leptin stimulation as the number of live basophils increased (data not shown). Time-course analyses revealed that leptin maintained basophil survival at each time point. These results indicate that leptin is a survival-enhancing factor for human basophils, exerting effects similar to those reported for it on other granulocytes, including eosinophils and neutrophils.

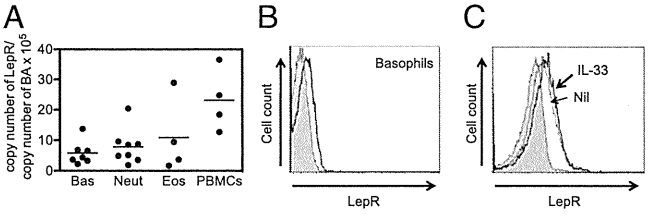

FIGURE 1. Expression of LepR by human basophils. *A*, cDNAs from highly purified basophils ($n = 8$), eosinophils ($n = 8$), neutrophils ($n = 4$), and PBMCs ($n = 4$) were tested for LepR by real-time quantitative PCR. The data are expressed as: (copy number of LepR gene/copy number of β-actin gene) × 100,000. Error bars show the mean quantity for each cell type. *B*, Surface expression of LepR by human basophils. Highly purified basophils were incubated with either anti-LepR Ab (bold line) or control Ab (shaded area) and then analyzed by flow cytometry. The data are representative of three separate experiments showing similar results. *C*, Surface expression of LepR by human basophils after overnight incubation. Highly purified basophils were incubated with (bold line) and without (thin line) IL-33 at 100 ng/ml, and then the surface expression of LepR was analyzed by flow cytometry. Shaded area shows basophils stained with control Ab. The data are representative of three separate experiments showing similar results.

Leptin positively regulates CD63 expression on human basophils

CD63 is expressed on the inner membrane of the cytoplasmic granules of human basophils, and it appears on the cell surface when the inner membrane fuses with the surface membrane at the time of degranulation. Therefore, it is thought that CD63 can be used as a marker of degranulation and activation of human basophils (32). Accordingly, we next studied the effect of leptin on human basophil expression of CD63 (Fig. 4*A*). IL-3 was used as a control and, consistent with an earlier report (33), it markedly upregulated CD63 expression (Fig. 4). Leptin also enhanced surface CD63 expression by basophils, and surprisingly, at 10 μM, this enhancement was much stronger than that by IL-3 at 100 pM. The effect of leptin was dose-dependent and was maximal at 10 μM. Importantly, the enhancing effect of leptin was neutralized by LepR blockade, as shown in Fig. 4*B*, indicating that leptin affects basophils through binding to LepR. Also, when we added a neutralizing Ab against leptin, the surface level of CD63 did not change (data not shown), implying that intrinsic leptin does not affect the innate CD63 level on basophils.

We also examined whether leptin affected human basophil surface expression of CD11b, another activation marker for human basophils and often an important adhesion molecule involved in basophil adhesion to the basement membrane and endothelial cells of vessel walls (34, 35). However, leptin showed almost no enhancement of CD11b expression on human basophils (data not shown).

Leptin affects degranulation of human basophils

Next, we tested whether leptin could directly induce degranulation of human basophils. We analyzed basophil degranulation by measuring the amount of histamine released in the supernatant. As a result, leptin at 10 μM directly induced basophil degranulation, although the effect was rather small, accounting for only 10% of total histamine (Fig. 5*A*). Importantly, leptin enhanced basophil degranulation triggered by CRA-1 Ab. A statistically significant effect was seen with leptin at 10 μM, reaching 10–20% of release enhancement (Fig. 5*B*). These data indicate that leptin has a small but significant direct effect on basophil degranulation, and that it primes basophils for an enhanced response to aggregation of FcεRI.

Leptin induces Th2 cytokine production by human basophils

One of the most important roles of human basophils is production of IL-4 and IL-13, key Th2 cytokines involved in IgE production and the initiation, formation, and exacerbation of allergic inflammation. Thus, we examined whether leptin affects the production of these cytokines by human basophils. As shown in Fig. 6*A*, mRNA levels for IL-13 were elevated by leptin. Also, the protein levels of Th2 cytokines, measured by ELISA, were higher in the case of leptin treatment (Fig. 6*B*), although the effects were

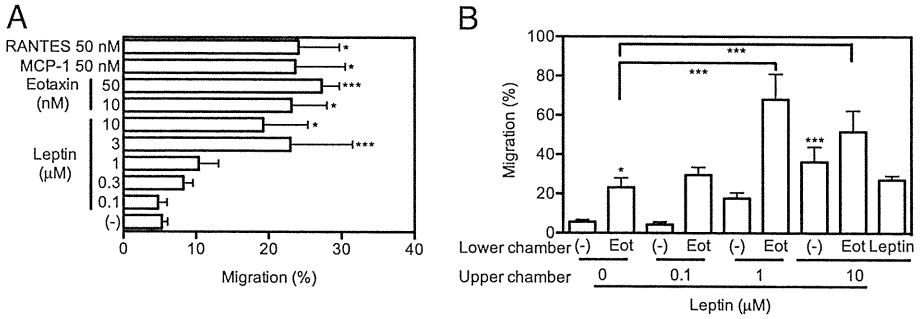

FIGURE 2. Leptin induces migration of human basophils. *A*, Twenty thousand Percoll-separated basophils were added to the upper chambers of Chemotaxicell, and the indicated concentrations of chemokines and leptin were added to the lower chambers. The percentages of migrated cells are shown. Error bars represent the SEM (n = 4–8). $*p < 0.05$, $***p < 0.001$ versus spontaneous migration in medium alone. *B*, Leptin enhances human basophil migration toward eotaxin. Cells mixed with the indicated concentrations of leptin were added to the upper chambers and then tested for migration toward eotaxin at 10 nM in the lower chambers. The percentages of migrated cells are shown. Error bars represent the SEM (n = 3–4). $*p < 0.05$, $***p < 0.001$ versus spontaneous migration in medium alone or corresponding migration toward eotaxin.

smaller than those of IL-3. Collectively, leptin showed a positive influence on Th2 cytokine production by basophils. In contrast, production of Th1 cytokines, specifically IL-2 and IFN-γ, by human basophils was quantified using a Luminex 200 (Luminex, Austin, TX) and Bio-Plex human cytokine assay kits (Bio-Rad Laboratories, Hercules, CA). These cytokines were below the limit of detection, and leptin thus seemed to have no apparent effect on Th1 cytokine production by basophils.

Leptin is expressed in human basophils

It has already been reported that human basophils produce and release leptin (36). To evaluate the capacity of human basophils to synthesize leptin, we performed real-time PCR analysis and ELISA of the basophil culture supernatant. Real-time PCR demonstrated that human basophils expressed mRNA for leptin, as previously reported (36). The expression level did not change in response to IL-33 (100 ng/ml) stimulation (RQ value to the expression level without stimulation, 0.96 ± 0.77; n = 4). In constrast, the concentrations of leptin in the supernatant of basophils cultured in medium alone, or stimulated overnight with IL-3 at 300 pM, IL-33 100 ng/ml, or anti-human IgE Ab at 10 μg/ml, did not reach the lower limit of detection of the ELISA kit (data not shown), indicating that the level of leptin produced by basophils is minimal.

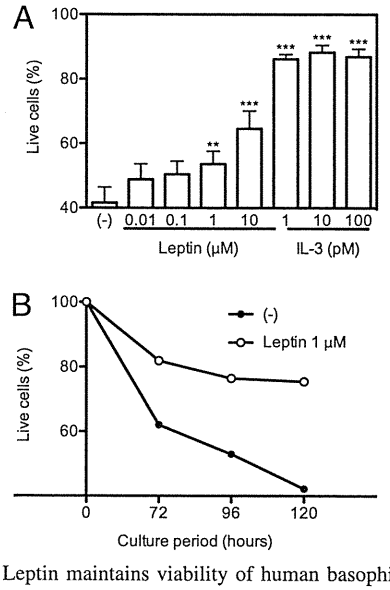

FIGURE 3. Leptin maintains viability of human basophils. Highly purified basophils were cultured in medium alone or in the presence of leptin or IL-3 at the indicated concentrations for 96 h. The cells were analyzed by double staining with annexin V and PI. *A*, Live cells were negative for both annexin V and propidium iodide. Data are expressed as percentages of total cell numbers. Error bars represent the SEM (n = 4). $**p < 0.01$, $***p < 0.001$ versus medium alone. *B*, Time course of human basophil survival. Highly purified basophils were cultured for the indicated periods of time and the percentages of live cells are shown. Data are representative of four separate experiments showing similar results.

Discussion

Leptin, along with TNF-α, IL-6, and IL-1, is one of the adipokines most abundantly produced by adipocytes that reside in white adipose tissue and whose primary roles are energy storage and regulation (37). Leptin not only acts as an important regulator of body weight but also shows other biological activities relating to hematopoiesis, angiogenesis, and immune responses (22). It is thought that elevated serum levels of leptin in obese individuals may contribute to chronic risk of developing inflammatory conditions (38). Also, there is accumulating evidence that leptin may play various roles in immunomodulation (20, 39). For example, leptin exerts a proliferative effect on $CD4^+$ T cells and also promotes production of proinflammatory Th1 cytokines such as IL-2 and IFN-γ by $CD4^+$ T cells (40–42). It is now widely accepted that cross-talk between inflammatory cells and adipocytes can be mediated by adipokines including leptin, and adipocytes are recog-

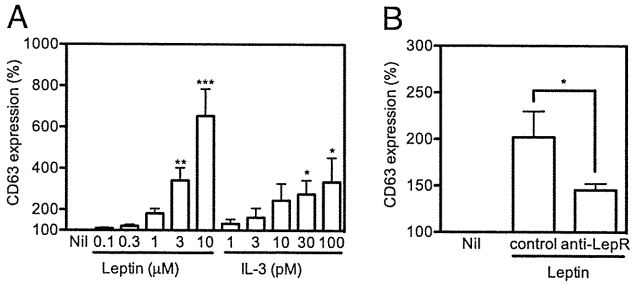

FIGURE 4. Leptin upregulates surface expression of CD63 by human basophils. *A*, Percoll-separated basophils were incubated with medium alone, leptin, or IL-3 at the indicated concentrations for 1 h. Then the surface CD63 expression level was assessed by flow cytometry. The data are expressed as the percentages of MESF values of basophils treated without any stimulus. Error bars represent the SEM (n = 4). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus nil. *B*, Percoll-separated basophils were incubated with leptin at 3 μM plus either control goat IgG or anti-LepR neutralizing Ab at 20 μg/ml and then stained for surface CD63 expression. The data are expressed as the percentages of MESF values of basophils treated without any stimulus. Error bars represent the SEM (n = 3). $*p <$ 0.05.

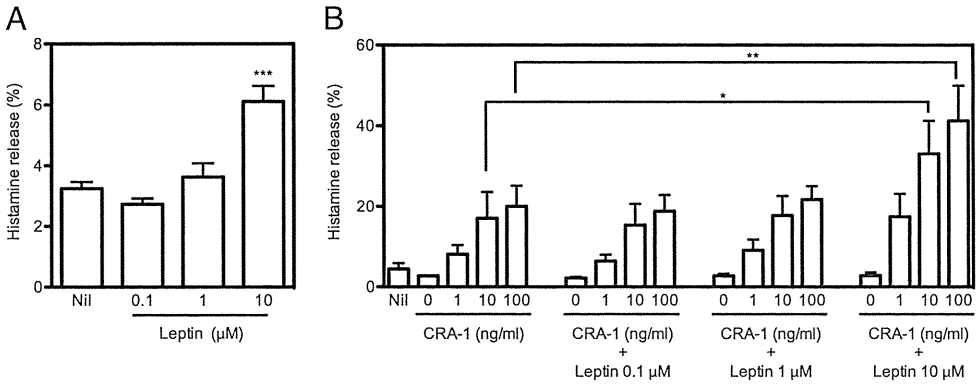

FIGURE 5. Leptin affects basophil degranulation. *A*, Percoll-separated basophils were stimulated with the indicated concentrations of leptin for 45 min, and degranulation was analyzed by measuring the histamine level in the supernatant. The percentage of release was calculated based on the total cellular histamine content. Error bars represent the SEM ($n = 5$). ***$p < 0.001$ versus spontaneous release of histamine in medium alone. *B*, Percoll-separated basophils were incubated with and without leptin at the indicated concentrations for 1 h and then stimulated with CRA-1 mAb at the indicated concentrations. Error bars represent the SEM ($n = 5$). *$p < 0.05$, **$p < 0.01$ versus the corresponding release without leptin pretreatment.

nized to participate in regulating immunological inflammation (43).

Our present study found that human basophils express mRNA for LepR at a level lower than that by PBMCs but similar to neutrophils and eosinophils. Both neutrophils and eosinophils have previously been shown to express LepR, which mediates the actions of leptin (23, 24). Also, freshly isolated basophils clearly expressed LepR protein on their surface. The fact that LepR expression on basophils was upregulated by IL-33, but not by IL-3 or IgE aggregation, seems to be a characteristic of this receptor. It was reported that human basophils express leptin (36), and we have confirmed that these cells express mRNA for leptin. However, their expression of LepR had not been demonstrated until now. Our findings indicate that exogenously added leptin potently affected various aspects of basophil activation, including enhanced migration, survival, CD63 expression, degranulation, and cytokine production, through binding to LepR.

Human basophils usually reside in the bloodstream, and when allergic inflammation is initiated, they migrate and accumulate at the inflammatory sites. We have been investigating the mechanisms that control basophil influx into the sites of inflammation. We previously demonstrated that specific Ags can attract basophils, which then use matrix metalloproteinases to move across the basement membrane (28, 34). Our present study revealed that leptin is also involved in basophil locomotion. It is noteworthy that the migration-inducing activity of leptin was as strong as those of two other known basophil chemoattractants, RANTES and eotaxin. In addition to its chemotactic effect, leptin showed an enhancing effect on migration toward eotaxin. These results collectively suggest that leptin is one of the most potent chemoattractants for human basophils, possessing both chemotactic and chemokinetic potential.

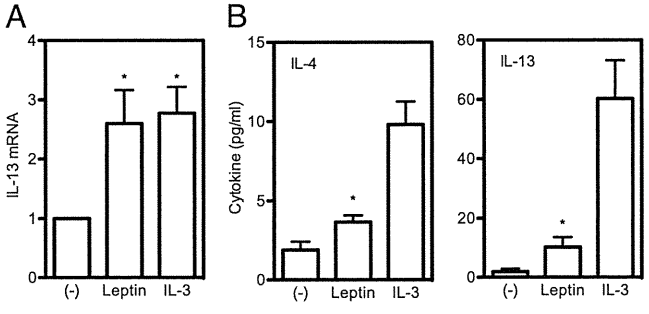

FIGURE 6. Leptin induces production of IL-4 and IL-13 by human basophils. *A*, Highly purified basophils were incubated with leptin at 10 μM or IL-3 at 100 pM for 24 h, and the expression levels of IL-13 mRNA by the cells were analyzed by real-time PCR. Data are expressed as the RQ values to the expression without stimulation. Error bars represent the SEM ($n = 5$). *$p < 0.05$ versus medium alone. *B*, Highly purified basophils (5×10^5/ml) were treated with leptin at 10 μM or IL-3 at 100 pM for 24 h, and then IL-4 and IL-13 in the supernatant were measured by ELISA. Error bars represent the SEM ($n = 5$–7). *$p < 0.05$ versus medium alone.

It has already been reported that leptin acts as a survival factor for eosinophils and neutrophils (23, 24). Our present study found that leptin is also a potent survival-enhancing cytokine for basophils. The fact that leptin attracts human basophils and prolongs their survival in vitro suggests that it may influence the development of allergic inflammation in the lungs, where leptin is known to be highly expressed. Moreover, there is increasing clinical evidence for a distinct role of leptin in airway diseases: the serum leptin level correlates positively with the severity of allergic rhinitis (44), increased leptin expression in the bronchial mucosa of chronic obstructive pulmonary disease patients is associated with airway inflammation and airflow obstruction (45), and serum leptin is elevated in female and child asthmatics (46, 47). These findings, together with our present results, suggest that leptin may act as a cytokine involved in the pathogenesis of airway inflammation by prolonging the lifespan of activated inflammatory cells, including basophils.

We also found intriguing results indicating that leptin strongly activates human basophils. First, the expression level of CD63 on the surface of basophils was strongly upregulated by leptin. CD63 is a membrane protein of the LAMP family, which is involved in vesicle fusion events and has been shown to be associated with release of cellular histamine (32). Our assessment of the amount of histamine released showed that basophil degranulation was greatly upregulated by leptin. Although direct induction of basophil degranulation by leptin was minimal, leptin potently primed basophils for enhanced degranulation in response to IgE or FcεRI aggregation. It has also been reported that splenocytes from histidine decarboxylase-deficient mice, which are deficient in histamine, produced larger amounts of leptin as well as IFN-γ and TNF-α in response to Ag stimulation (48). Collectively with our present data, the evidence suggests that leptin may be deeply involved in histamine homeostasis. In addition to upregulation of basophil degranulation, leptin enhanced Th2 cytokine production by human basophils, whereas it showed no effect on their Th1 cytokine production. Basophils are known to be one of the most

potent cell types secreting IL-4 and IL-13, and our data suggest that leptin will accelerate the initiation of Th2-mediated inflammation by activating basophils to produce IL-4 and IL-13. It is remarkable that leptin may upregulate Th2 responses by enhancing basophil Th2 cytokine production, since leptin has long been thought to promote Th1 responses rather than Th2 responses (40). These results imply the existence of an underlying mechanism whereby leptin induces exacerbation of allergic diseases.

The effect of leptin on human basophil activation seems to occur only at high concentrations. The biological levels of leptin found in venous blood under physiological conditions are usually 1–100 ng/ml. However, leptin serum levels rise up to 400 ng/ml in children with chronic renal failure (49), and high levels of leptin are also found in extremely obese subjects due to leptin resistance (50). Additionally, serum leptin levels >700 ng/ml were reported in obese individuals treated with leptin (51). Therefore, leptin may reach levels in vivo that can induce basophil activation under certain circumstances. It is suspected that leptin levels induced in inflamed tissue sites may be even higher than the levels in serum. Also, the low reactivity of basophils seen in experiments might be due to the relatively low potency of recombinant leptin compared with native leptin because of the difference in its glycosylation pattern (52).

Although we confirmed that human basophils express mRNA for leptin, they did not produce large amount of leptin by themselves. Thus, basophils may not be a major source of leptin, but leptin released by them may contribute to paracrine effects on other inflammatory cell types.

Epidemiologic data indicate that the serum levels of leptin are increased in patients suffering from atopic asthma and IgE-associated atopic eczema (47). Also, obesity increases the prevalence of asthma, worsens asthma control, lowers the quality of life, and increases asthma-related hospitalizations (13, 53). In fact, weight loss in obese asthmatics reduces airway obstruction and results in improved lung function, symptoms, morbidity, and health status (16). Our results suggest that the link between obesity and allergic inflammation may be due, at least in part, to leptin-mediated cross-talk between adipocytes and basophils. Besides its potent effects on basophils, leptin shows pleiotropic effects on various types of inflammation, including Th2-mediated reactions. Leptin promotes proliferation of human monocytes (54) and NK cells (55) and their production of inflammatory cytokines protects neutrophils and eosinophils from apoptosis (23, 24) and promotes T cell activation (39). Also, leptin is reported to negatively modulate regulatory T cell proliferation and function (56, 57), which may lead to increased severity of inflammation due to decreased immunological tolerance. In summary, at the sites of allergic inflammation observed in asthma, leptin may act as a potentially important mediator capable of perpetuating airway inflammation by attracting, prolonging the survival of, and activating the functions of various inflammatory cells, including human basophils.

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Disclosures

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