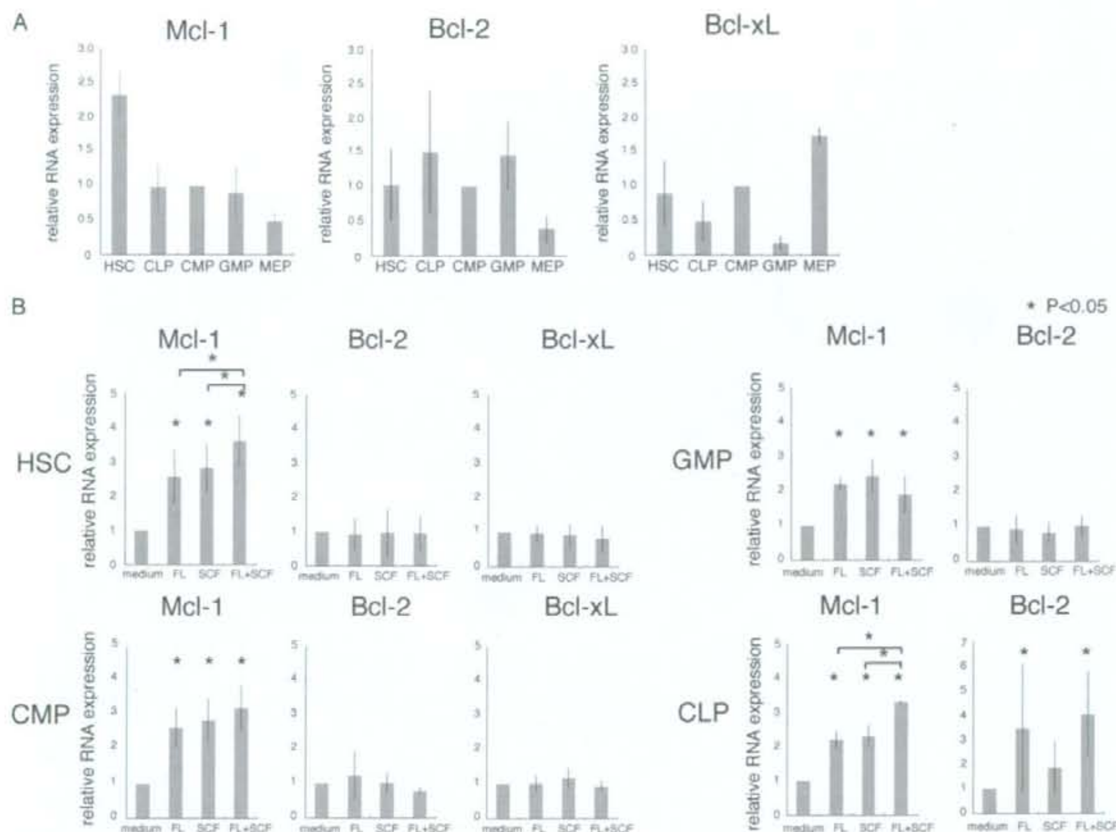

FIGURE 6. *A*, Quantitative RT-PCR assays for human anti-apoptotic genes such as Mcl-1, Bcl-2, and Bcl-x_L in purified HSCs and each progenitor population. Each bar represents an *n*-fold difference in the amount of anti-apoptotic gene expression relative to that in Flt3$^+$ CMPs. Note that Mcl-1 expression level is highest in HSCs, whereas Bcl-2 and Bcl-x_L expression is most pronounced in GMPs and MEPs, respectively. *B*, Changes in anti-apoptotic gene expression in each progenitor after incubation with FL and/or SCF. Significant up-regulation of Mcl-1 mRNA was seen in HSCs, Flt3$^+$ CMPs, GMPs, and CLPs after incubation with FL and/or SCF. Each bar represents the mean value and the SD of sixe independent samples.

CMPs formed a variety of myelo-erythroid colonies including clonogenic CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), whereas hFlt3$^-$ CMPs did not form CFU-GEMM, but preferentially differentiated into the MegE lineage. Since GMPs (hFlt3$^+$) and MEPs (hFlt3$^-$) exclusively gave rise to GM- and MegE-related colonies, respectively, hFlt3 expression could be associated with GM lineage development. These results suggested that hFlt3$^+$ CMPs might differentiate into MEPs via hFlt3$^-$ CMPs. We thus directly tested the lineage relationship of these purified myelo-erythroid progenitor populations (Fig. 4*B*). hFlt3$^+$ and hFlt3$^-$ CMPs were purified and cultured in vitro. Then, 72 h after the initiation of culture, hFlt3$^+$ CMPs gave rise to hFlt3$^-$ CMPs, hFlt3$^+$ GMPs and hFlt3$^-$ MEPs, whereas hFlt3$^-$ CMPs did not up-regulate hFlt3, differentiating only into hFlt3$^-$ MEPs. Such phenotypically defined secondary myeloid progenitors displayed differentiation activity consistent with their phenotypic definition (Fig. 4*C*). These data suggest that multipotent hFlt3$^+$ CMPs can differentiate into both GMPs and MEPs, whereas hFlt3$^-$ CMPs represent a transitional stage into MEPs.

Flt3 signaling protects human hematopoietic stem and progenitor cells from apoptotic cell death

We wished to elucidate the role of Flt3 signaling in human hematopoiesis. We first tested the effect of Flt3 signaling on the differentiation of HSCs, CMPs, and GMPs. Purified hFlt3$^+$ HSCs, CMPs, and GMPs were cultured in methylcellulose in the presence of the myeloid cytokine mixture, with or without hFL. As shown in Fig. 5*A*, the addition of FL in the culture did not affect the percentage of GM, MegE, or mix colonies in any of these populations. Interestingly, however, the colony numbers significantly increased in all cases when FL was added to the culture. This effect was dose-dependent, and the stimulatory activity of FL reached its peak at a concentration of 5 ng/ml (not shown). The plating efficiencies of hFlt3$^+$ HSCs, CMPs, and GMPs cultured with the cytokine mixture containing FL (20 ng/ml) were significantly higher than those cultured without FL, suggesting that FL signaling may enhance the viability of cells (Fig. 5*A*). We then directly tested the viability of HSCs, CMPs, and GMPs 24 h after the initiation of culture in serum-free media, with or without FL. The live, apoptotic, and dead cells after culture were enumerated by the Annexin/PI staining (43). In this staining, live cells are Annexin$^-$/PI$^-$, whereas Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells are apoptotic and dead cells, respectively (Fig. 5*B*). Without FL, a considerable proportion of purified HSCs, CMPs, and GMPs rapidly became Annexin$^+$/PI$^-$ and Annexin$^+$/PI$^+$ cells undergoing apoptotic cell death. The addition of FL significantly blocked apoptotic cell death in all of these populations, indicating that FL plays a critical role in human hematopoietic stem and progenitor cell survival (Fig.

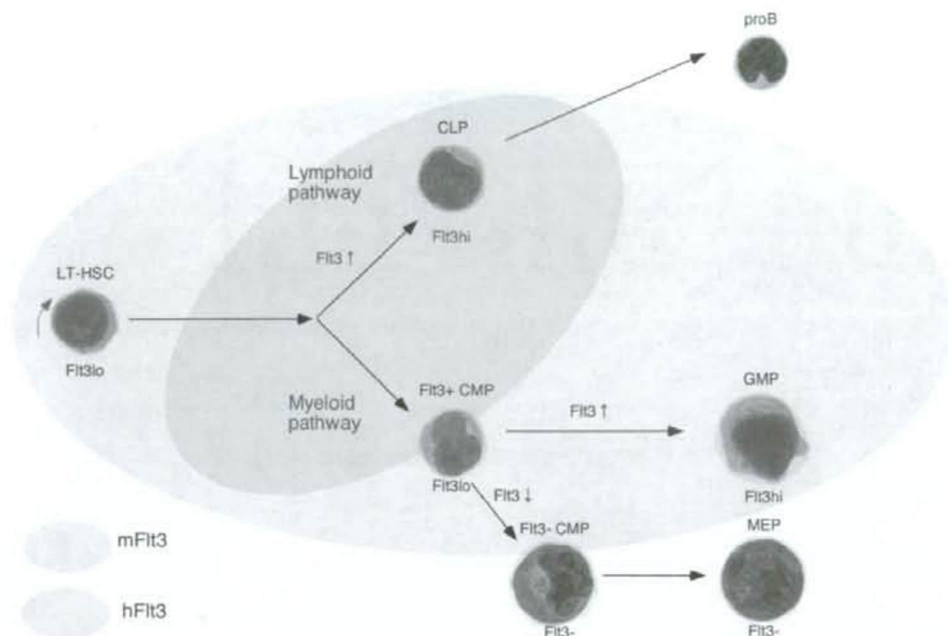

FIGURE 7. Proposed differential expression of human and mouse Flt3 in steady-state hematopoiesis. Cellular morphology of directly sorted each progenitors (May-Giemsa ×1000) is shown here. In human, the most primitive LT-HSC expressed hFlt3 at a low level and its expression is up-regulated at the early GM and the lymphoid progenitor stages, while it is down-regulated in MEPs. In contrast, the mouse LT-HSC lacks mFlt3 expression, and mFlt3 is expressed in cells primed to the lymphoid pathway, including CLPs and a fraction of CMPs.

5*B*). These data strongly suggest that Flt3 signaling does not instruct hematopoietic lineage commitment in hFlt3-expressing myeloid progenitors, but it does promote their survival.

SCF, the ligand for c-Kit, has also been shown to play a critical role in the maintenance of survival in early hematopoiesis. Both c-Kit and Flt3 belong to the class III receptor tyrosine kinase (RTK) family, sharing their major signaling cascade (44). Human HSCs, CMPs, and GMPs expressed both c-Kit and Flt3 at the single cell level (Fig. 1). Thus, we tested the anti-apoptotic effect of SCF in this system. As shown in Fig. 5*C*, in all HSC, CMP, and GMP populations, SCF also displayed anti-apoptotic effects whose impact on cell survival is similar to that of FL. Furthermore, in HSCs and CMPs, the combination of FL and SCF further increased percentages of live cells as compared with those in the presence of either FL or SCF alone, suggesting that SCF and FL signals collaborate to maintain cell survival of HSCs and CMPs.

Flt3 signaling up-regulates Mcl-1, but not Bcl-2 or Bcl-x_L, expression in human hematopoietic stem and progenitor cells

The question: is the mechanism of cell survival enhancement by signaling of RTKs, such as Flt3 and c-Kit? We have shown that in murine hematopoiesis, Mcl-1, a Bcl-2 homologue, is indispensable for hematopoietic stem and progenitor cell survival, and that c-Kit signaling is one of the most critical inducers for Mcl-1 expression in mHSCs (45). We therefore hypothesized that Flt3, as well as c-Kit, signaling may up-regulate Mcl-1 to maintain cell survival in human hematopoiesis as well.

Fig. 6*A* shows the distribution of the transcripts of Bcl-2 family molecules including Mcl-1, Bcl-2, and Bcl-x_L in human stem and progenitor cells. Mcl-1 is expressed at the highest level in HSCs. CMPs and CLPs expressed similar levels of Mcl-1, and MEPs expressed Mcl-1 at the lowest level. This expression pattern of Mcl-1 transcript in human hematopoiesis is consistent with that in murine hematopoiesis (45). In contrast, Bcl-2 was highly expressed in GMPs and CLPs, whereas Bcl-x_L was expressed in MEPs at the highest level.

Purified stem and progenitor populations were incubated with FL and/or SCF in serum-free media. Both FL and SCF dramatically up-regulated the expression of Mcl-1 in a dose-dependent manner, and it reached its peak 30 min after initiation of culture at a concentration of 5 ng/ml (data not shown). Fig. 6*B* shows the relative expression level of Mcl-1, Bcl-2, and Bcl-x_L in the presence of 20 ng/ml FL and/or SCF. We found that both FL and SCF significantly up-regulated the expression of Mcl-1, but not of Bcl-2 or Bcl-x_L, in HSCs, CMPs, and GMPs. These data collectively suggest that one of the important functions of these class III RTKs is to specifically activate Mcl-1 expression. Interestingly, in HSCs, FL and SCF displayed an additive effect on the up-regulation of Mcl-1. Therefore, Flt3 and c-Kit signaling collaborate to protect $Flt3^+$ HSCs and early myeloid progenitors from apoptotic cell death, presumably through activating anti-apoptotic Mcl-1 transcription. In CLPs, however, FL activated not only Mcl-1 but also Bcl-2 transcription.

Discussion

In this study, by using a multicolor FACS and a highly efficient xenograft system, we provide evidence that the distribution of Flt3 RTK is quite different in human and mouse hematopoiesis. First, although mouse LT-HSCs do not express mFlt3, the HSC-enriched $hCD34^+hCD38^-hLin^-$ population, that can reconstitute human hematopoiesis for a long-term in our xenogenic mouse model, uniformly expresses hFlt3 in both BM and CB. It is still unclear whether SCID-repopulating cells directly correspond to hLT-HSCs. However, because the $hCD34^+hCD38^+hLin^-$ cells never

reconstituted in xenogenic hosts for a long-term in our and others' experiments (42), it is highly likely that $hCD34^+hCD38^-hLin^-$ population is highly enriched for hLT-HSCs. Therefore, it is suggested that the negative expression of hFlt3 does not mark LT-HSCs in human, while mFlt3 does in mouse (16, 17). Second, in contrast to mouse hematopoiesis, where mFlt3 expression is restricted within progenitor populations of lymphoid potential including CLPs and a minority of CMPs that can differentiate into B cells (20), hFlt3 is expressed in human CMPs and GMPs, as well as in CLPs. The Flt3 expression is suppressed after cells are committed into the MegE lineage in both human and mouse. The distribution of Flt3 in mouse and human hematopoiesis is schematized in Fig. 7. The significant difference of Flt3 distribution in human and mouse hematopoiesis suggests that the critical role of Flt3 signaling in hematopoietic development could also be different between these species.

We further found that the important function of hFlt3 should include the maintenance of cell survival via the up-regulation of anti-apoptotic Mcl-1 in early hematopoiesis. Previous studies have demonstrated that FL can support in vitro survival of human long-term culture-initiating cells (24, 46, 47). MCL-1 is a non-redundant anti-apoptotic protein, at least in mouse hematopoiesis, because the removal of Mcl-1 from hematopoietic cells in a conditional knockout system caused fatal hematopoietic failure, and because in vitro disruption of *Mcl-1* in mouse HSCs, CMPs, or CLPs rapidly induced their apoptotic cell death (45). The expression level of Mcl-1 was the highest at the HSC stage and gradually declined as HSCs differentiate into myeloid and lymphoid progenitors in mouse hematopoiesis (45). The pattern of Mcl-1 distribution is well preserved in human hematopoiesis (Fig. 6*A*), suggesting that Mcl-1 might also be essential for hHSC survival. In mouse HSCs, Mcl-1 is up-regulated by signals from cytokines including SCF, IL-6, and IL-11, and SCF exerts the most potent effect on the up-regulation of Mcl-1(45). In contrast to mouse LT-HSCs that express c-Kit but not Flt3, functional hLT-HSCs coexpress c-Kit and Flt3 (Fig. 1), and importantly, FL as well as SCF are potent inducers for Mcl-1 transcription (Fig. 6). The fact that FL and SCF activated only Mcl-1, but not Bcl-2 or Bcl-x_L, in turn suggests that Mcl-1 might be the most critical survival factor controlled by exogenous cytokine signals at the HSC stage. Although it remains unclear whether hFlt3 and/or c-Kit signaling is absolutely required for hHSC survival, our data suggest that, to maintain the Mcl-1 level in hHSCs, the Flt3/FL system could work as an alternative to the SCF/c-Kit system. This is of interest because the SCF/c-Kit system is non-redundant in mouse hematopoiesis (48), where mouse LT-HSCs express only c-Kit, but not Flt3.

The anti-apoptotic effect of hFlt3 signaling was also seen in hFlt3-expressing myeloid progenitor populations. The incubation of CMPs and GMPs with FL significantly prevented their apoptotic cell death in vitro, and FL, as well as SCF, rapidly activated the Mcl-1 transcription in these progenitors. Interestingly, in CLPs, FL activated not only Mcl-1 but also Bcl-2. In lymphopoiesis, Bcl-2 (49, 50), as well as Mcl-1 (51), is critical. FL may collaborate with IL-7 to maintain lymphoid cell survival by up-regulating both Bcl-2 and Mcl-1. Collectively, in humans, Flt3 signaling might support cell survival in early hematopoietic stages with only the exception of the MegE lineage developmental pathway.

Our data also provides an important insight into pathogenesis of AML with *FLT3* mutations. A total of 15–35% of AML patients have either internal tandem duplications (ITDs) in the juxtamembrane domain or mutations in the activating loop of *FLT3* (28, 29), resulting in ligand-independent constitutive signal activation. The *FLT3* mutations are rarely found in acute lymphoblastic leukemia (28, 29). The etiologic link of *FLT3* mutations with AML does not fit the lymphoid-only expression pattern of Flt3 in mouse hematopoiesis. In mouse models, however, the ectopic expression of *FLT3*-ITDs in the bone marrow promotes development of myeloproliferative disorders, but these mutations themselves do not cause leukemia (52). We have found that AML cells with *FLT3*-ITD mutations possess extremely high levels of Mcl-1, and transduction of *FLT3*-ITD into normal HSCs induces rapid up-regulation of Mcl-1 of up to >10-fold higher levels (G. Yoshimoto and K. Akashi, manuscript in preparation). Because the expression of *FLT3* mutations should occur in concert with that of normal Flt3, our data suggest that once *FLT3* mutations are acquired in human hematopoiesis, abnormal survival-promoting signals of Mcl-1 should be expressed in LT-HSCs, and is progressively up-regulated in GMPs. It has been shown that both LT-HSCs and GMPs are the critical cellular target for leukemic transformation. The reinforced survival of CMPs/GMPs by blocking two independent apoptotic pathways (53), or the enforced expression of bcr-abl together with survival-promoting Bcl-2 at the GMP stage (54), results in AML development in mouse models. In human bcr-abl-positive chronic myelogeneous leukemia, GMPs could be the target for blastic transformation by acquisition of β-catenin signaling (55). GMPs can also be converted into leukemic stem cells simply by transducing leukemia fusion genes, such as MLL-ENL (56) or MOZ-TIF2 (57). Thus, these data collectively suggest that the acquisition of *FLT3* mutations in human hematopoiesis might induce the reinforced survival of cells at the HSC and myeloid progenitor stages, where *FLT3* mutations might collaborate with other genetic abnormalities to achieve full AML transformation.

In conclusion, our data show that the distribution of Flt3 is quite different in mouse and human hematopoeisis. hFlt3 targets LT-HSCs and myeloid progenitors except for MEPs. Flt3 signaling might support cell survival in early hematopoiesis including the HSC and the myeloid progenitor stages through up-regulation of Mcl-1. This is a striking example that the expression pattern of key molecules could be significantly different between human and mouse. Accordingly, special considerations are required in using mouse models to understand the role of Flt3 and *FLT3* mutations in human hematopoiesis.

Disclosures

The authors have no financial conflict of interest.

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ARTICLE

Identification of the human eosinophil lineage-committed progenitor: revision of phenotypic definition of the human common myeloid progenitor

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To establish effective therapeutic strategies for eosinophil-related disorders, it is critical to understand the developmental pathway of human eosinophils. In mouse hematopoiesis, eosinophils originate from the eosinophil lineage-committed progenitor (EoP) that has been purified downstream of the granulocyte/macrophage progenitor (GMP). We show that the EoP is also isolatable in human adult bone marrow. The previously defined human common myeloid progenitor (hCMP) population (Manz, M.G., T. Miyamoto, K. Akashi, and I.L. Weissman. 2002. *Proc. Natl. Acad. Sci. USA*. 99:11872–11877) was composed of the interleukin 5 receptor α chain$^+$ (IL-5Rα$^+$) and IL-5Rα$^-$ fractions, and the former was the hEoP. The IL-5Rα$^+$CD34$^+$CD38$^+$IL-3Rα$^+$CD45RA$^-$ hEoPs gave rise exclusively to pure eosinophil colonies but never differentiated into basophils or neutrophils. The IL-5Rα$^-$ hCMP generated the hEoP together with the hGMP or the human megakaryocyte/erythrocyte progenitor (hMEP), whereas hGMPs or hMEPs never differentiated into eosinophils. Importantly, the number of hEoPs increased up to 20% of the conventional hCMP population in the bone marrow of patients with eosinophilia, suggesting that the hEoP stage is involved in eosinophil differentiation and expansion in vivo. Accordingly, the phenotypic definition of hCMP should be revised to exclude the hEoP; an "IL-5Rα–negative" criterion should be added to define more homogenous hCMP. The newly identified hEoP is a powerful tool in studying pathogenesis of eosinophilia and could be a therapeutic target for a variety of eosinophil-related disorders.

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Abbreviations used: BaP, basophil lineage-committed progenitor; CCR3, CC chemokine receptor 3; CMP, common myeloid progenitor; EoP, eosinophil lineage-committed progenitor; Epo, erythropoietin; EPX, eosinophil peroxidase; FOG-1, friend of GATA-1; GMP, granulocyte/macrophage progenitor; HDC, histidine decarboxylase; HES, hypereosinophilic syndrome; HSC, hematopoietic stem cell; IL-5Rα, IL-5R α chain; MegE, megakaryocyte/erythroid; MEP, megakaryocyte/erythrocyte progenitor; MNC, mononuclear cell; MPO, myeloperoxidase; MPP, multipotent progenitor; PMN, polymorphonuclear cell; SCF, stem cell factor; Tpo, thrombopoietin.

Eosinophils mainly reside in the gastrointestinal mucosa and normally constitute only 1–5% of blood nucleated cells. Eosinophils play an important role in host defense against parasitic infections and are major effectors in a variety of allergic reactions (1, 2). Upon diverse stimuli, infiltrating eosinophils cause chronic inflammatory tissue damage by releasing a wide spectrum of proinflammatory mediators, including major basic protein, eosinophil cationic protein, and eosinophil-derived neurotoxin (2, 3).

Like other hematopoietic lineages, eosinophils originate from the hematopoietic stem cell (HSC). In mouse hematopoiesis, the eosinophil lineage-committed progenitor (EoP) (4) exists as a distinct population downstream of the granulocyte/macrophage progenitor (GMP) (5). The mEoP expressed the receptor for IL-5 that plays an important role in controlling eosinophil numbers (6–8). The mouse bipotent basophil/mast cell progenitor and the basophil

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lineage-committed progenitor (BaP) were also identified downstream of the mGMP (9), suggesting that the commitment of eosinophil and basophil/mast cell lineages independently occurs after the multipotent progenitor (MPP) has lost a megakaryocyte/erythroid (MegE) lineage potential. In contrast, in human hematopoiesis, cells possessing both basophil and eosinophil granules have been found in leukemia patients (10, 11), suggesting the close relationship between the basophil and the eosinophil lineages. The early works using methylcellulose colony assays of human bone marrow cells demonstrated that eosinophils were scattered preferentially within erythroid (12) or myeloid (13) colonies. These data raise the possibility that the eosinophil developmental pathway is considerably different between the human and mouse. However, such retrospective assessments of progenitor functions based on lineage readouts in colony assays does not necessarily reflect their full lineage potential because MPPs could decide lineage fates in a random manner, at least in vitro (14).

In this paper, we identified the EoP in human bone marrow. The previously defined human common myeloid progenitor (hCMP) (15) was divided into the IL-5R α chain$^+$ (IL-5Rα$^+$) and IL-5Rα$^-$ fractions, and the former was fully committed to the eosinophil lineage lacking the MegE, the neutrophil/monocyte, and the basophil/mast cell potentials. The hEoP was generated from the IL-5Rα$^-$ hCMP but not from the hGMP or the human megakaryocyte/erythrocyte progenitor (hMEP). Thus, the prospectively purified hEoP represents the initial stage of eosinophil development independent of the hGMP, the hMEP, the putative human basophil/mast cell progenitor, or the hBaP. Interestingly, the hEoP population expanded significantly in the bone marrow of patients with eosinophilia, suggesting that the hEoP might be a promising therapeutic target for eosinophil-related allergic and inflammatory disorders. These data led us to revise our original definition of the hCMP population by using "IL-5Rα-negative" as an additional phenotypic criterion.

RESULTS

Three granulocyte subclasses such as neutrophils, eosinophils, and basophils can be purified from human blood by FACS

Isolation of a lineage-committed progenitor population by FACS is highly dependent on lineage-specific surface markers. In mouse hematopoiesis, eosinophils but not basophils or neutrophils possessed a high level of IL-5Rα, and the expression of IL-5Rα was a key to isolate the mEoP (4). We first tested the expression of IL-5Rα on human blood leukocytes and found that both eosinophils and basophils expressed IL-5Rα at high levels. We further found that eosinophils and basophils independently resided within the polymorphonuclear cell (PMN) gate and the mononuclear cell (MNC) gate, respectively (Fig. 1 A). Thus, by combinatory analysis of the expression pattern of IL-5Rα and of the forward scatter versus the side scatter cytogram, three classes of human blood granulocytes became clearly visible: IL-5Rα$^-$ PMNs were neutrophils, IL-5Rα PMNs were eosinophils, and IL-5Rα MNCs were basophils (Fig. 1 A). The purity of sorted populations was >99% in all of these fractions.

Fig. 1 B shows the expression patterns of cell-surface molecules related to the eosinophil and/or the basophil/mast cell lineages. CC chemokine receptor 3 (CCR3), a high-affinity receptor for eotaxin that promotes the migration of eosinophils and basophils into the inflammatory tissue (16, 17), was expressed on both purified eosinophils and basophils. β7 integrin is an essential molecule for tissue-specific homing of precursors for mouse intestinal mast cells (18), and is expressed on mouse mast cells and basophils but not on eosinophils (9, 19). In human blood, however, not only basophils but also eosinophils expressed β7 integrin. CD203c, the ectonucleotide pyrophosphatase/phosphodiesterase 3, is reported to be a marker for human basophils, mast cells, and their precursors (20). In our hands, however, CD203c was expressed on not only basophils but also eosinophils, indicating that this molecule is not specific for the basophil/mast cell lineage. Thus, none of these markers appeared to be useful to differentiate human eosinophils from basophils on FACS, except for a high-affinity receptor for IgE α chain (FcεRIα) that was expressed only on human basophils (Fig. 1 B).

We further tested the gene expression of lineage-specific markers in each FACS-purified granulocyte subclass. The expression of myeloperoxidase (MPO), eosinophil peroxidase (EPX), and histidine decarboxylase (HDC) transcripts was evaluated. As shown in Fig. 1 C, human neutrophils exclusively possessed MPO but lacked the expression of EPX or HDC. Human eosinophils and basophils did not express MPO but possessed only EPX and HDC, respectively. Thus, in addition to the morphological analysis, the evaluation of these functional molecules should be useful to differentiate eosinophils from basophils and neutrophils.

Eosinophils develop from hHSCs and hCMPs but not from hGMPs or hMEPs

To delineate the developmental origin of human eosinophils, we tested the eosinophil lineage readout of myeloid progenitor populations in steady-state bone marrow. As shown in Fig. 2 A, the lineage-affiliated antigen (Lin$^-$) and CD34$^+$ human bone marrow MNC fraction was subdivided into the CD38$^-$ hHSC and the CD38$^+$ progenitor populations (21). The Lin$^-$CD34$^+$CD38$^+$ human progenitors were further fractionated into hCMP, hGMP, and hMEP populations according to the expression patterns of IL-3Rα and CD45RA, as we previously reported (15): hCMPs, hGMPs, and hMEPs were visualized as the IL-3Rα$^+$CD45RA$^-$, IL-3Rα$^+$CD45RA$^+$, and IL-3Rα$^-$CD45RA$^-$ populations, respectively (Fig. 2 A). In liquid cultures supplemented with a cytokine cocktail containing stem cell factor (SCF), IL-3, IL-5, GM-CSF, erythropoietin (Epo), and thrombopoietin (Tpo), hHSCs and hCMPs generated CCR3-expressing eosinophils/basophils as well as other myelomonocytic and MegE cells, whereas hGMPs and hMEPs gave rise mainly to neutrophils/monocytes/macrophages and MegE cells, respectively, without developing into the eosinophil lineage (Fig. 2 B). Basophils were also scattered

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in the progeny of hHSCs, hCMPs, and hGMPs but not of hMEPs (unpublished data). In agreement with FACS and morphological analyses, progeny of hHSCs and hCMPs expressed both the eosinophil-affiliated EPX and the basophil-affiliated HDC transcripts in addition to the MPO transcript, whereas progeny of hGMPs or hMEPs did not express EPX

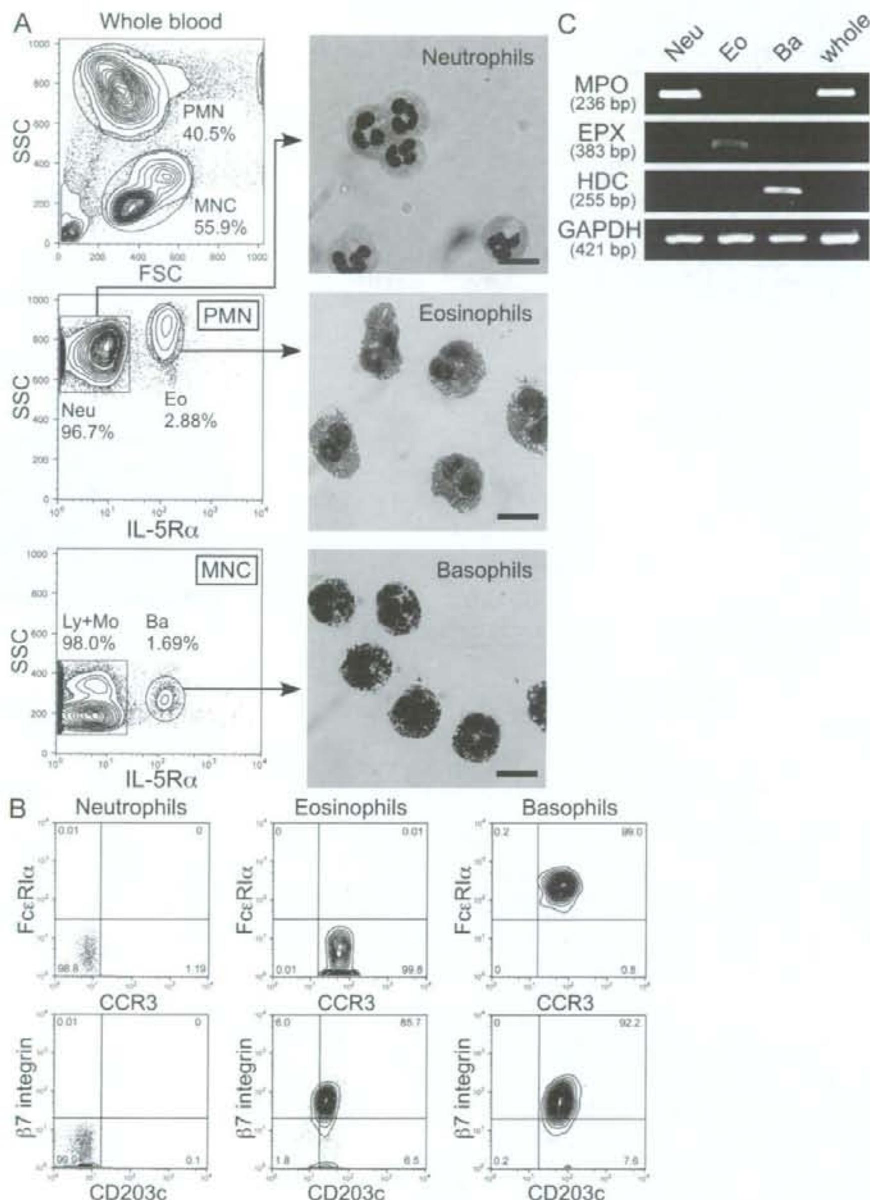

Figure 1. Purification of neutrophils, eosinophils, and basophils. (A) Sorting gates for three granulocyte subclasses. The representative FACS plots of normal blood leukocytes are shown. IL-5Rα⁻ and IL-5Rα⁺ fractions within the PMN gate were neutrophils (Neu) and eosinophils (Eo), respectively. Basophils (Ba) resided within the MNC gate and expressed IL-5Rα at a high level. The purity of each sorted population was >99%, as determined morphologically by cytospin preparations (May-Giemsa staining). Bars, 10 µm. (B) FACS analyses of cell-surface molecules that relate to the eosinophil and/or the basophil lineages on each granulocyte subclass (percentages are shown). (C) RT-PCR analyses of lineage-affiliated gene expression in FACS-purified granulocyte subclasses (whole, whole blood leukocytes). Data were reproducible in two independent analyses using different blood samples. FSC, forward scatter; SSC, side scatter.

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(Fig. 2 C). The HDC transcript was also detected in progeny of hGMPs but not of hMEPs (Fig. 2 C). We further performed methylcellulose assays, where we picked up all single colonies and made cytospin preparations to morphologically define cell components. As shown in Fig. 2 D, ~3% of hHSC- and ~5% of hCMP-derived colonies contained eosinophils. hGMPs and hMEPs never generated eosinophil-containing colonies. More interestingly, ~4% of hCMP colonies were composed only of eosinophils (Fig. 2 D), suggesting that a fraction of cells within the hCMP gate may be progenitors already restricted to the eosinophil lineage.

The IL-5Rα⁺ fraction within the original hCMP is the hEoP

To separate the putative hEoP, we tested the expression of IL-5Rα in stem and progenitor populations. The expression of IL-5Rα was detectable only in the hCMP fraction: ~10% of hCMPs expressed IL-5Rα (Fig. 3 A). The IL-5Rα$^+$ fraction of hCMPs also expressed very low levels of CCR3 and β7 integrin (not depicted) that were detected on blood mature eosinophils and basophils at high levels (Fig. 1 B). FcεRIα was specifically expressed on blood basophils (Fig. 1 B) but not detected in the hCMP population (not depicted). Strikingly, in methylcellulose assays, purified IL-5Rα$^+$ hCMPs gave rise only to pure eosinophil colonies (Fig. 3, B and C). In contrast, the IL-5Rα$^-$ fraction of hCMPs generated a variety of myeloid colonies including rare (~2%) eosinophil-containing colonies (Fig. 3 B). In liquid cultures, the day 14 progeny of IL-5Rα$^+$ hCMPs significantly up-regulated CCR3 on their surface (Fig. 3 D). These cells possessed EPX but not MPO or HDC transcripts, whereas progeny of IL-5Rα$^-$ hCMPs

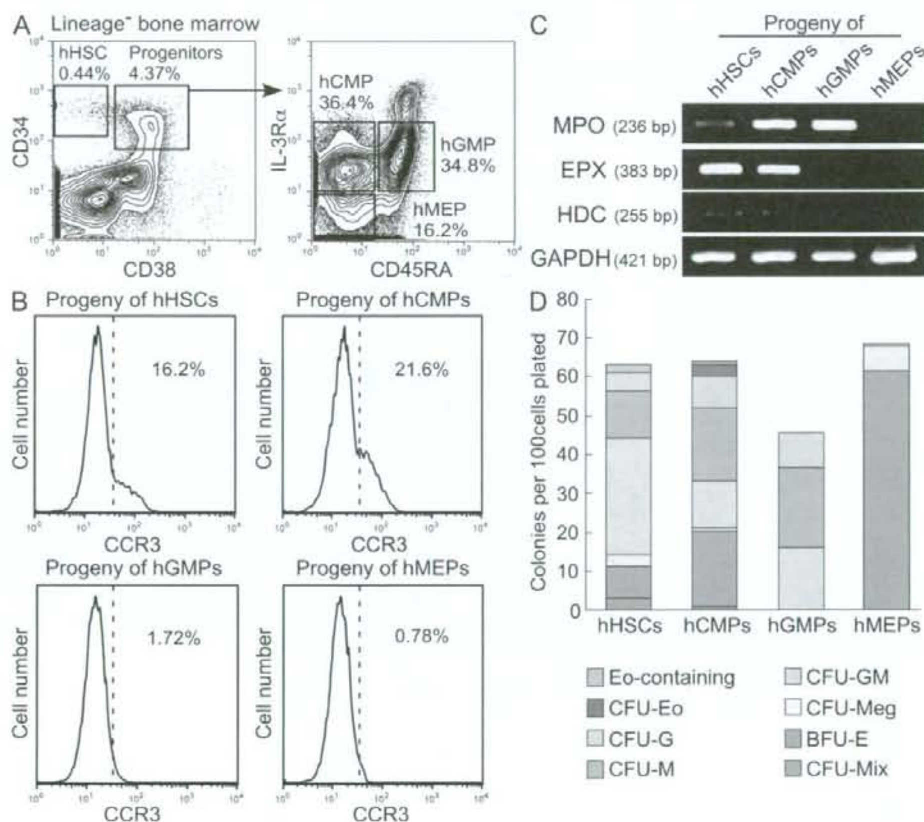

Figure 2. Eosinophils develop from hHSCs and hCMPs but not from hGMPs or hMEPs. (A) Sorting gates for hHSC and downstream myeloid progenitor populations. (B) The emergence of CCR3⁺ eosinophils in cultures of FACS-purified progenitor populations. A cytokine cocktail used in this experiment contained SCF, IL-3, IL-5, GM-CSF, Epo, and Tpo. CCR3⁺ eosinophils were detected in hHSC and hCMP cultures but not in hGMP or hMEP cultures. Dashed lines indicate cut-off lines of negative controls. (C) RT-PCR analyses of lineage-affiliated gene expression in the progeny of each progenitor population. The expression of eosinophil-specific EPX was detected in progeny of hHSCs and hCMPs but not of hGMPs or hMEPs. (D) The methylcellulose colony assays of FACS-purified progenitor populations. The eosinophil-containing (Eo-containing) colonies developed from hHSCs and hCMPs but not from hGMPs or hMEPs. Approximately 4% of hCMP-derived colonies were composed only of eosinophils (CFU-Eo). Data were reproducible in two independent experiments using different bone marrow samples.

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expressed all of these molecules (Fig. 3 E), supporting the eosinophil lineage-restricted potential of the IL-5Rα⁺ hCMP population. These observations collectively suggest that the IL-5Rα⁺ fraction of original hCMP has committed to the eosinophil lineage. Thus, the hEoP was as isolatable as the IL-5Rα⁺hCD34⁺hCD38⁺IL-3Rα⁺hCD45RA⁻ population in steady-state human bone marrow. These data also in turn show that the original hCMP (15) includes the hEoP contaminant, and that its phenotypic definition should be revised as IL-5Rα⁻hCD34⁺hCD38⁺IL-3Rα⁺hCD45RA⁻.

hEoPs develop from hCMPs independent of hGMPs and hMEPs

The question was of the lineage relationship between hEoPs and other myeloid progenitors. We tried to track the emergence of hEoPs within the culture of IL-5Rα⁻ hCMPs and other myeloid progenitors. The hCMPs gave rise to a minor fraction of progeny expressing IL-5Rα on day 5 (Fig. 4 A), together with hGMPs and hMEPs (not depicted). The IL-5Rα⁺ progeny gave rise exclusively to pure eosinophil colonies (Fig. 4 B), and they possessed the surface phenotype identical to that of hEoPs (Fig. 4 C). Neither hGMPs nor hMEPs generated hEoPs at any time point during the culture (unpublished data). These data strongly suggest that the hEoPs develop from IL-5Rα⁻ hCMPs but not from hGMPs.

Consistent with their capability of functional readouts, IL-5Rα and EPX transcripts were exclusively expressed in hEoPs, whereas other eosinophil-specific genes such as Charcot-Leyden crystal protein and major basic protein were

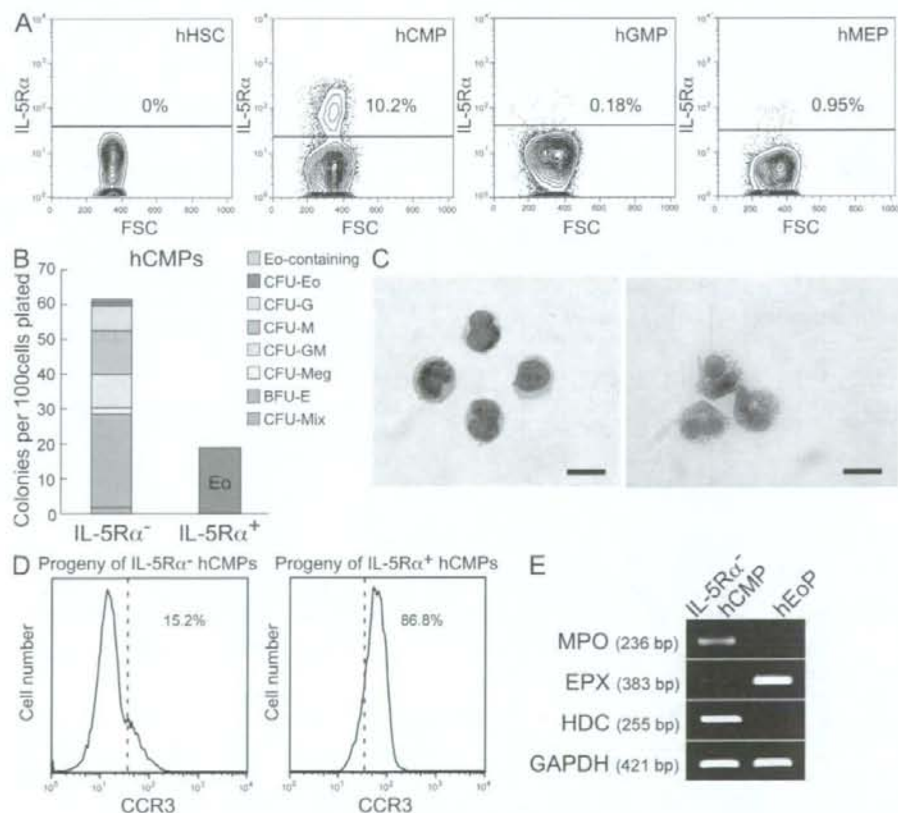

Figure 3. The IL-5Rα⁺ fraction within the original hCMP represents the hEoP. (A) FACS analysis of IL-5Rα expression on myeloid progenitor populations. The representative FACS plots of normal bone marrow progenitors are shown. (B) The methylcellulose colony assays of IL-5Rα⁻ and IL-5Rα⁺ fractions within the hCMP. The cytokine cocktail used in this experiment was the same as in Fig. 2 B. All of the single colonies were picked up and mounted on cytospin preparations to define cell components. The IL-5Rα⁺ fraction of hCMP only generated pure eosinophil colonies (CFU-Eo). (C) The morphologies of purified hEoPs and their progeny. hEoPs represented a blastic morphology with fine cytoplasmic granules (left), and their progeny were composed only of eosinophils (right; May-Giemsa staining). Bars, 10 µm. (D) FACS analysis of CCR3 expression on the progeny of IL-5Rα⁻ hCMPs and hEoPs. Dashed lines indicate cut-off lines of negative controls. (E) RT-PCR analyses of lineage-affiliated gene expression in progeny of IL-5Rα⁻ hCMPs and hEoPs. The progeny of hEoPs expressed a high level of EPX but never possessed MPO or HDC transcripts. Data were reproducible in two independent experiments using different bone marrow samples.

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expressed at low levels in hCMPs but were up-regulated at the hEoP stage (Fig. 5 A). hMEPs and hGMPs never expressed any of these eosinophil-related molecules (Fig. 5 A).

Expression profiles of lineage-instructive transcription factors in hEoPs

In mouse hematopoiesis, GATA-1 and GATA-2 play critical roles in the eosinophil lineage development. GATA-1–deficient mice lack eosinophils (22, 23), and the enforced expression of GATA-1 or GATA-2 instructed both mouse and human myeloid progenitors to develop into the eosinophil lineage (23–25). As shown in Fig. 5 B, hEoPs possessed approximately two- to threefold increased levels of GATA-1 and GATA-2 as compared with those in hCMPs, whereas hGMPs completely shut down these GATA factors. The expression level of GATA-1 in hEoPs was significantly lower than that of hMEPs, whereas the GATA-2 level in hEoPs was higher than that in hMEPs (Fig. 5 B). Friend of GATA-1 (FOG-1) is a critical cofactor of GATA family transcription factors and plays an indispensable role in the mouse MegE lineage development (26, 27), whereas it antagonizes the eosinophil development presumably interfering with eosinophil lineage-related functions of GATA factors (28). Consistent with these mouse data, hEoPs significantly down-regulated the FOG-1 expression, whereas hMEPs expressed FOG-1 at the highest level (Fig. 5 B). The distribution of these GATA family transcription factors in human myeloid progenitors was identical to that in mouse counterparts (29).

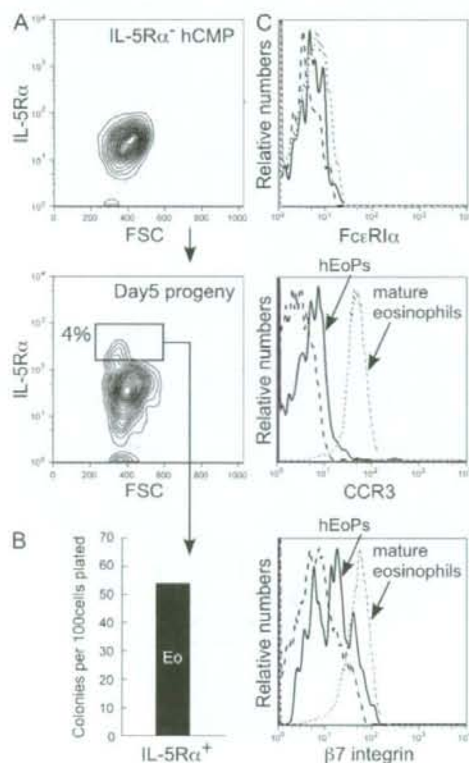

Figure 4. hEoPs develop from hCMPs independent of hGMPs and hMEPs. (A) Development of IL-5Rα⁺ hEoPs from IL-5Rα⁻ hCMPs in liquid culture. (B) The IL-5Rα-expressing cells purified from the primary hCMP culture formed only eosinophil colonies in the presence of the multiple cytokines used in Fig. 2 B. All single colonies were picked up and were mounted on cytospin preparations to define cell components. (C) FACS analyses of cell-surface molecules on the hEoP (continuous line, hEoPs; dashed line, blood eosinophils; bold dashed line, control antibodies). Data were reproducible in two independent experiments using different bone marrow samples. FSC, forward scatter.

C/EBPα is required for the development of mGMPs as well as their progeny, including eosinophils (30). In our hands, in mouse hematopoiesis, the eosinophil lineage commitment can be activated by up-regulation of GATA-2 in the presence of C/EBPα (29). As shown in Fig. 5 B, the expression level of C/EBPα was highest in hGMPs but was diminished in hMEPs. Importantly, hCMPs, which are immediate precursors of hEoPs, possessed a significant amount of C/EBPα, and hEoPs maintained its expression with up-regulation of GATA-1 and GATA-2. PU.1 is indispensable for mouse granulocyte and lymphoid cell development (31, 32). The expression of PU.1 was detected in both hGMPs and hEoPs but was suppressed at the hMEP stage.

These data show that changes in expression patterns of lineage-instructive transcription factors in these myeloid progenitors are similar between human and mouse hematopoiesis (29). Therefore, the developmental machinery of eosinophil lineage might be well preserved between these species.

The hEoP population significantly expanded in patients with eosinophilia

To evaluate whether the hEoP contributes toward eosinophil production in vivo, we enumerated the number of hEoPs in the bone marrow of eosinophilia patients. Patients' characteristics are summarized in Table I. In normal bone marrow samples, the hEoP population accounted for only 0.033% of bone marrow MNCs or 2.38% of Lin⁻CD34⁺ cells, respectively (Table I and Fig. 6 A). We analyzed 15 patients with eosinophilia: 5 patients with hypereosinophilic syndrome (HES), 2 patients with T cell malignancies, 1 chronic myelogenous leukemia patient in accelerated phase, 2 patients with Churg-Strauss syndrome, and 5 eosinophilia patients with unknown etiology. The *FIP1L1/PDGFRα* fusion gene was not detected in any of these patients. The blood eosinophil count was 13,799 μl, and eosinophil lineage cells accounted for 38.4% of whole bone marrow cells on average (Table I). The bone marrow hEoP in eosinophilia patients consisted of 0.125% of MNCs or 7.44% of Lin⁻CD34⁺ cells, respectively (Table I and Fig. 6 A). Thus, eosinophilia patients possessed three- to fourfold higher numbers of hEoPs compared with normal controls ($P < 0.01$; Fig. 6 A). These data suggest that the hEoP stage is actively involved in generation of eosinophils in patients with eosinophilia.

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DISCUSSION

In the present study, we identified the hEoP in steady-state human bone marrow. This population gave rise to eosinophils but not neutrophils or basophils in vitro. It possessed the eosinophil-specific EPX transcript but did not have the basophil-specific HDC or the neutrophil-specific MPO transcripts. Furthermore, the hEoP significantly expanded in number in the bone marrow of patients with eosinophilia. Thus, like mouse hematopoiesis, the developmental pathway initiating from the distinct hEoP stage exists in human bone marrow, and the hEoP should actively contribute toward generation of eosinophils in vivo.

We have also developed a method to ultimately purify mature neutrophils, eosinophils, and basophils from human

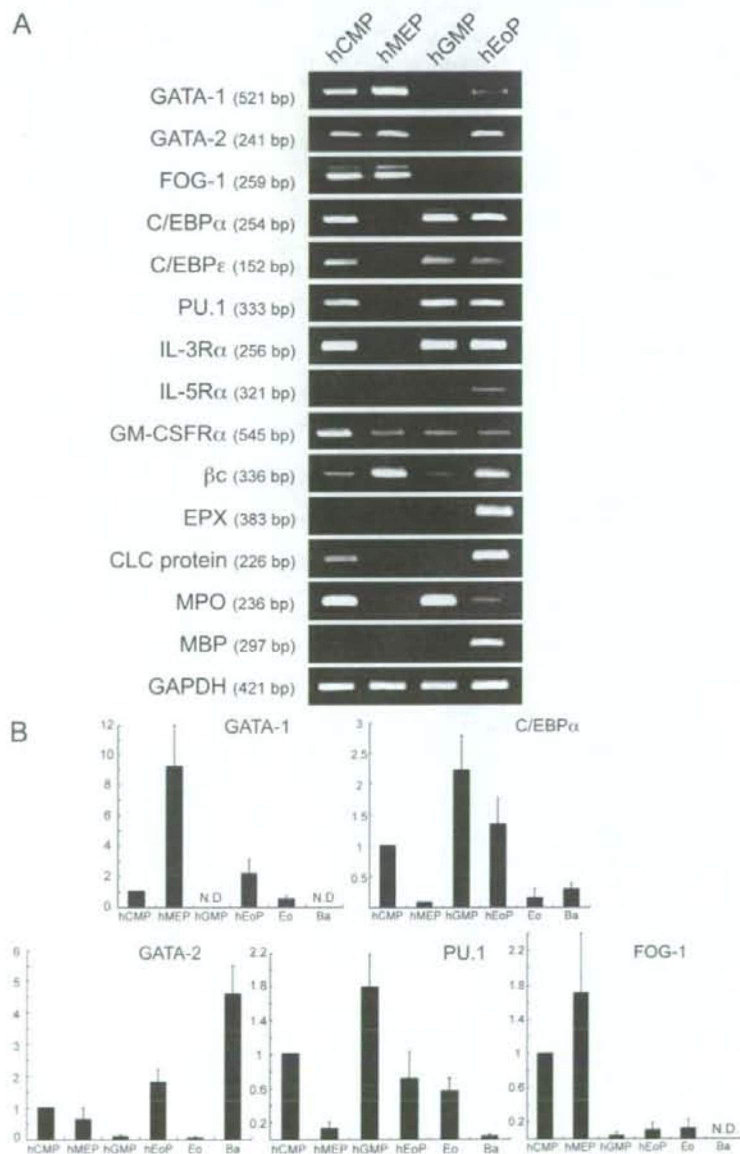

Figure 5. The comparison of lineage-affiliated gene expression between hEoPs and other myeloid progenitors. (A) RT-PCR analyses of lineage-affiliated genes. (B) Quantitative real-time PCR analyses of lineage-instructive transcription factors (Ba, blood basophils; Eo, blood eosinophils). Data were reproducible in three independent analyses using different RNA samples. Data represent means ± SEM.

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blood (Fig. 1 A). In contrast to mouse hematopoiesis, where IL-5Rα is expressed in the eosinophil but not the basophil lineage, mature human basophils expressed IL-5Rα at a level comparable to that of eosinophils (Fig. 1 A). Interestingly, the IL-5Rα$^+$CD34$^+$CD38$^+$ population (i.e., hEoP) gave rise only to eosinophils and activated the EPX, whereas they did not differentiate into basophils or neutrophils nor did they activate the HDC or the MPO after culture (Fig. 3). We found that the hEoP having the identical IL-5Rα$^+$CD34$^+$CD38$^+$ phenotype resided also in the umbilical cord blood and in the adult peripheral blood. They were extremely rare populations (~0.005% of cord blood and 0.001% of blood MNCs, respectively) but differentiated only into eosinophils at a similar efficiency (unpublished data). Thus, the IL-5Rα$^+$CD34$^+$CD38$^+$ phenotype is common to the hEoP in multiple locations, including the bone marrow, the peripheral blood, and the cord blood. hEoPs may emigrate from the bone marrow into circulation and may be recruited into sites of allergic inflammation.

The expression of IL-5Rα in hEoPs might occur as a result of eosinophil lineage commitment without exerting instructive functions for eosinophil lineage specification. We have previously shown that the enforced IL-5 signaling at the mGMP stage by transducing mouse IL-5Rα induced mainly neutrophil/macrophage- but not eosinophil-specific differentiation (4). Consistent with this data, hEoPs generated only eosinophils even when we removed IL-5 from the cytokine cocktail, suggesting that IL-3 and/or GM-CSF signaling is sufficient to support the survival/proliferation and the terminal maturation of hEoPs. In addition, G-CSF, a cytokine that promotes neutrophil differentiation, did not affect the fate of hEoPs. It corresponds with our finding that hEoPs lack the expression of G-CSF receptor transcript (unpublished data). These observations collectively suggest that the hEoP is absolutely committed to the eosinophil lineage irrespective of a cytokine milieu.

Basophils were produced from populations within the IL-5Rα$^-$CD34$^+$CD38$^+$ fraction, such as hCMPs and hGMPs, as well as the CD34$^+$CD38$^-$ hHSCs (Fig. 2 C). Therefore, in normal human hematopoiesis, the IL-5Rα$^+$CD34$^+$CD38$^+$ hEoP does not contain progenitors common to eosinophils and basophils. It is still unclear at what stage basophils up-regulate IL-5Rα. Because all IL-5Rα$^+$CD34$^+$CD38$^+$ cells have committed to the eosinophil lineage, and because hGMPs were capable of producing basophils (Fig. 2 C), putative hBaP may exist within or downstream of the hGMP population. Basophils may up-regulate IL-5Rα at a later stage of their maturation after cells shut off CD34 expression. To isolate hBaPs, the identification of new surface antigens that mark early basophil lineage commitment is necessary.

This study also indicates that the conventional hCMP population defined by our earlier work (15) was heterogeneous because it contained at least the hEoP. Therefore, we should revise our original definition of hCMP: the true hCMP (i.e., revised hCMP) resides in the IL-5Rα$^-$ fraction of conventional hCMP (Fig. 6 B). The IL-5Rα$^-$ hCMP gave rise to the IL-5Rα$^+$ hEoP (Fig. 4), suggesting that the eosinophil

Table I. Percentages of hEoP in normal and eosinophilia bone marrow

Patient no.	Diagnosis	Age/sex	WBC (/μl)	Eosinophils in PB (%)	Eosinophils in BM (%)	hEoP in BMMNCs (%)	hEoP in Lin$^-$CD34$^+$ (%)
1	HES	17/M	18,510	12,957 (70)	NA	0.227	7.72
2	HES	50/M	29,530	14,647 (49.6)	NA	0.253	6.87
3	HES	51/M	13,220	4,680 (35.4)	21.2	0.186	3.85
4	HES	62/F	16,800	9,593 (57.1)	36.4	0.047	12.7
5	HES	68/M	12,800	3,430 (26.8)	32.8	0.048	4.73
6	T cell lymphoma	75/F	30,180	27,011 (89.5)	66.8	0.039	11.8
7	ATL	60/M	57,510	24,442 (42.5)	38	0.056	5.66
8	CML	48/M	436,000	34,008 (7.8)	9.6	0.33	8.46
9	AGA	34/F	23,210	14,669 (63.2)	49.6	0.074	5.82
10	AGA	66/F	40,000	33,240 (83.1)	76.4	0.072	9.91
11	unknown	81/F	9,300	2,632 (28.3)	36.4	0.043	7.56
12	unknown	26/F	10,970	5,331 (48.6)	22.4	0.052	3.84
13	unknown	50/F	13,610	3,062 (22.5)	28.8	0.076	8.67
14	unknown	57/M	21,020	13,747 (65.4)	37.6	0.207	6.92
15	unknown	76/M	10,380	3,529 (34)	43.2	0.161	7.09
Average		57	49,536	13,799 (48.3)	38.4	0.125	7.44
SD		(17–81)	107,711	11,026 (23.2)	18.1	0.094	2.61
Normal BM (n = 7)							
Average		46	5,164	176 (3.26)	3.09	0.033	2.38
SD		(28–72)	1,652	110 (1.21)	1.17	0.013	0.46

AGA, allergic granulomatous angitis; ATL, adult T cell leukemia; BMMNC, bone marrow MNC; CML, chronic myelogenous leukemia; NA, not applicable; PB, peripheral blood.

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lineage commitment occurs at least within the IL-5Rα⁻ hCMP stage. However, we cannot exclude the possibility that a portion of hEoPs develop directly from the earlier progenitors such as hHSCs and hMPPs, bypassing the hCMP stage. The hGMP does not faithfully correspond to the mGMP because the hGMP lacked the eosinophil potential (Fig. 2), whereas the mGMP was capable of producing all granulocyte subclasses, including eosinophils and basophils via their progenitor populations such as the mEoP and the mBaP. Therefore, it is also possible that the true hGMP with all granulocyte subclass potentials may reside within the IL-5Rα⁻ hCMP fraction (Fig. 6 B). Another possibility is that a putative erythroid/eosinophil progenitor that preferentially differentiates into the erythroid and the eosinophil lineages (12) may exist upstream of the hEoP within the IL-5Rα⁻ hCMP fraction independent of the hEoP, hMEP, and hGMP. To fully understand the myeloerythroid lineage relationship, these possibilities should be tested in future studies.

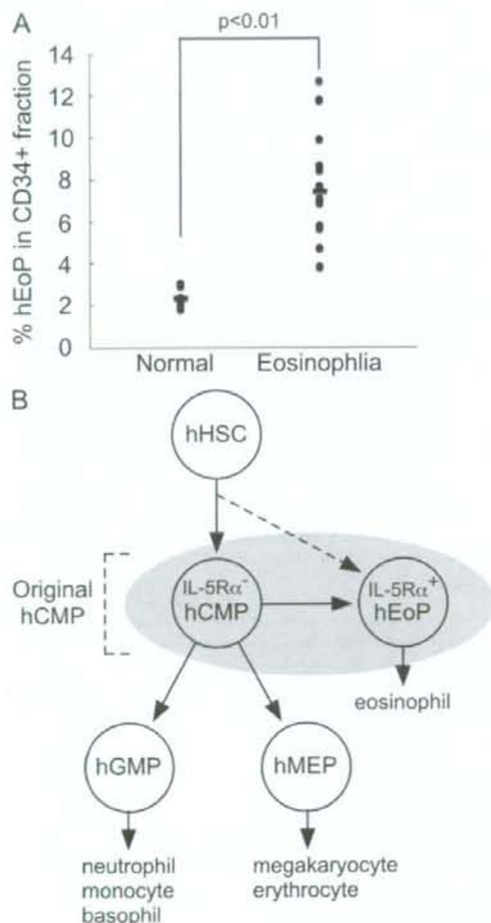

Figure 6. The in vivo expansion of hEoP in the bone marrow of eosinophilia patients. (A) The percentage of the hEoP population in the bone marrow CD34⁺ fraction. Eosinophila patients possessed approximately fourfold increased numbers of hEoPs (horizontal lines indicate means). Patients' characteristics are summarized in Table I. (B) The lineal relationship between the hEoP and other myeloid progenitor populations. The original hCMP (shaded) contained the hEoP. The revised hCMP is defined as the IL-5Rα⁻ fraction of the original hCMP. The hEoP develops from the hCMP or its upstream MPP independent of the hGMP and the hMEP.

Although the eosinophil developmental pathway could be somewhat different between the human and mouse, the use of key transcription factors appeared to be well preserved between these species (Fig. 5) (29). The hCMP expressed only low levels of GATA-1 and GATA-2 but both were upregulated in the hEoP. The expression of C/EBPα was maintained, whereas FOG-1 was suppressed in the hEoP. In mouse hematopoiesis, we have shown that the order of expression of C/EBPα and GATA-2 plays a pivotal role in the eosinophil versus the basophil lineage commitment (29). It is of interest whether such interplay of transcription factors is also critical in human hematopoiesis.

In summary, we have identified the hEoP as an IL-5Rα⁺ fraction of conventional hCMP. Thus, we propose to revise our original definition of hCMP by excluding an IL-5Rα⁺ fraction from the previous one: the revised phenotype of hCMP is IL-5Rα⁻Lin⁻CD34⁺CD38⁺IL-3Rα⁺CD45RA⁻. The hEoP significantly expands in patients suffering from diseases with eosinophilia, presumably reflecting their contribution toward eosinophil production in vivo. Therefore, this population should be useful to study the normal and abnormal eosinophil development, and could be a therapeutic target of diseases with eosinophila such as HES and allergic disorders.

MATERIALS AND METHODS

Bone marrow and blood samples. Bone marrow samples were obtained from healthy volunteers ($n = 7$) and eosinophilia patients ($n = 15$) by a conventional bone marrow aspiration procedure. All donors gave informed consent, and the study was approved by the Institutional Review Board of Kyushu University Hospital. Patients' characteristics are summarized in Table I. The diagnosis of HES was made according to the World Health Organization criteria. The *FIP1L1/PDGFRα* fusion gene, which relates to the pathogenesis of chronic eosinophilic leukemia, was analyzed by a nested RT-PCR method, as previously described (33). The EoL-1 cell line was used as a positive control for the PCR assay. All patients' samples analyzed in this study were negative for the *FIP1L1/PDGFRα* fusion gene.

Antibodies, cell staining, and sorting. Anti-human IL-5Rα monoclonal antibodies (KM1266) (34) were biotinylated with an EZ-Link NHS-PEO, Solid Phase Biotinylation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. To separate mature granulocyte subclasses, heparinized whole blood was stained with biotinylated anti-IL-5Rα antibodies followed by streptavidin-PE (eBioscience) in combination with anti-CCR3 (R&D Systems), anti-FcεRIα (eBioscience), anti-CD203c (Beckman Coulter), or anti-β7 integrin (BD) antibodies. The sorting procedures for hHSCs and myeloid progenitor populations that we previously reported (15) were slightly modified. In brief, bone marrow MNCs obtained by a density-gradient centrifugation method were first stained with PE-Cy5-conjugated lineage antibodies, including anti-CD3, -CD4, -CD8, -CD10, -CD19, -CD20, -CD14, -CD56, and -glycophorin A. Subsequently, cells were stained with allophycocyanin-conjugated anti-CD34 (BD), PE-conjugated anti-CD38 (Invitrogen),

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PE-Cy7–conjugated anti–IL-3Rα (eBioscience), and FITC-conjugated anti-CD45RA (eBioscience) antibodies. hHSCs, hCMPs, hGMPs, and hMEPs were isolated as Lin$^-$CD34$^+$CD38$^-$, Lin$^-$CD34$^+$CD38$^+$IL-3Rα$^+$CD45RA$^-$, Lin$^-$CD34$^+$CD38$^+$IL-3Rα$^+$CD45RA$^+$, and Lin$^-$CD34$^+$CD38$^+$IL-3Rα$^-$CD45RA$^-$ populations, respectively. To sort hEoPs and redefined hCMPs, Pacific blue–conjugated anti-CD38 antibodies were used, and biotinylated anti–IL-5Rα antibodies followed by streptavidin-PE were added. hEoPs and hCMPs were purified as Lin$^-$CD34$^+$CD38$^+$IL-3Rα$^+$CD45RA$^-$IL-5Rα$^+$ and Lin$^-$CD34$^+$CD38$^+$IL-3Rα$^+$CD45RA$^-$IL-5Rα$^-$ populations, respectively. Dead cells were excluded by propidium iodide staining. Appropriate isotype-matched control monoclonal antibodies were used to determine the background staining level in each channel. All sorting and analyses were performed on three laser-equipped FACSAria machines (BD). To minimize contamination, the second round of sorting was performed routinely with the same sorting gates as the first round. The automatic cell-deposition system was used for single-cell assays. FACS data were analyzed with FlowJo software (Tree Star, Inc.).

Cell culture. For liquid cultures, purified progenitor populations were suspended in 12-well plates with the following medium: IMDM (Invitrogen) supplemented with 20% FCS (StemCell Technologies Inc.), antibiotics, 20 ng/ml of human recombinant IL-3, 20 ng/ml IL-5, 20 ng/ml SCF, 50 ng/ml GM-CSF, 4 U/ml Epo, and 20 ng/ml Tpo (R&D Systems). For clonogenic analyses of hHSCs and myeloid progenitors, including hEoPs, cells were cultured 14 d in IMDM-based methylcellulose medium (Methocult H4100; StemCell Technologies Inc.) with 20% FCS, 1% BSA, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (StemCell Technologies Inc.). The same cytokines described were added at the initiation of cultures. All cultures were incubated at 37°C in a humidified chamber under 5% CO_2. All single colonies were picked up and were mounted on cytospin preparations to define cell components.

Gene expression analysis. Total RNA was extracted from purified progenitor populations, cultured cells, or mature blood granulocytes using ISOGEN reagent (Nippon Gene) according to the manufacturer's protocol. All RNA samples were reverse transcribed with Oligo dT primers using the SuperScript III First-Strand Synthesis System (Invitrogen). The conventional RT-PCR and the quantitative real-time PCR assays were performed with the GeneAmp 9700 PCR System and the PRISM 7500 Fast Real-Time PCR System, respectively (Applied Biosystems). The specific primer and probe sequences for PCR analyses are provided in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20081756/DC1). Human β2 microglobulin transcript was simultaneously amplified as an internal standard for quantification.

Online supplemental material. The specific primer and probe sequences for PCR analyses are listed in Table S1. Online supplemental material is available at available at http://www.jem.org/cgi/content/full/jem.20081756/DC1.

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ORIGINAL ARTICLE: CLINICAL

Validation of the revised 2008 WHO diagnostic criteria in 75 suspected cases of myeloproliferative neoplasm

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Abstract

The objective of this study was to validate the recently revised 2008 WHO diagnostic criteria of myeloproliferative neoplasms (MPN) together with the analysis of correlation of *JAK2 (Janus kinase 2)*-V617F mutant allele burden with clinical/laboratory findings on each patient. We made a diagnosis of 75 suspected MPN patients based on both diagnostic criteria of the 2001 WHO classification and the revised 2008 WHO classification, and found that both criteria show a quite similar diagnostic power except for two patients (idiopathic erythrocytosis (IE) and thrombocytosis) who were diagnosed as essential thrombocythemia by the 2008 WHO criteria. From *JAK2*-V617F analysis, hemoglobin and hematocrit values were significantly higher and platelet count was lower in *JAK2*-V617F high allele burden group than *JAK2*-V617F middle allele burden group. Mutant allele burden of polycythemia vera (PV) group was higher than that of essential thrombocythemia group. Therefore, the amount of mutant allele seemed to define the disease phenotypes. We further found a PV case presenting a rare type of *JAK2*-exon12 mutation. In contrast, IE presented a good prognosis unlike MPN. Hereafter, the 2008 WHO criteria with *JAK2* gene analysis are useful for precise diagnosis of MPN and the patients with erythrocytosis.

Keywords: *Myeloproliferative neoplasm, JAK2 mutation, genotyping*

Introduction

Clinical categories of chronic myeloproliferative diseases (CMPD) have recently been replaced by myeloproliferative neoplasms (MPN) in the 4th edition of the WHO classification of hematological neoplasms [1]. In 2005, the point mutation of *Janus kinase 2 (JAK2)* gene was reported on *bcr-abl*-negative MPN [2–8]. Primarily *JAK2*-V617F mutation is invariably associated with polycythemia vera (PV) and considered to be a responsible gene for the disease etiology [4,6,7]. However, the same *JAK2* mutation is detected in about a half of patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF) [5–9].

JAK2-V617F mutation occurs at one allele (the heterozygous pattern) or both alleles (the homozygous pattern) in PV patients, and previous studies have reported that *JAK2*-V617F homozygous group represents higher hemoglobin concentration, higher incidence of myelofibrotic change than *JAK2*-V617F heterozygous group [6,10,11]. Among MPN patients with *JAK2* mutation, the ratio of *JAK2*-V617F mutant (T) allele to *JAK2* total allele (V617F mutant allele plus wild type *JAK2* allele) is suggested to involve the clinical phenotypes of MPN [6,10,12–14]. In addition, novel mutations in the exon12 of *JAK2* gene have been reported on the *JAK2*-V617F-negative PV and idiopathic erythrocytosis (IE) patients [15–17], but such mutations are

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not detected either on ET or PMF patients [15,18,19].

In these contexts, we attempted to validate the recently revised WHO diagnostic criteria of MPN, and we paid attention to the mutant (T) allele burden of *JAK2* gene of the patients with MPN and analysed the relation of the amount of mutant allele expression with clinical phenotypes, laboratory data and therapeutic status according to the revised 2008 WHO diagnostic criteria. In addition, we have examined *JAK2*-exon12 mutations in *JAK2*-V617F-negative PV and IE patients, and found one case with a rare type of exon12 mutation.

Materials and methods

Patients and clinical data

The patients who visited our hospital and were diagnosed as or suspected of MPN from 2006 to 2007 entered this study. The whole study had been approved by the ethical committee of Kawasaki Medical School, and the samples or data of each patient were handled after informed consent. Clinical diagnosis was done according to both diagnostic criteria of the 2001 WHO classification and the revised 2008 WHO classification [1]. Clinical/laboratory findings of the first visit were utilised for the present study.

JAK2-V617F genotyping and allele burden analysis

JAK2-V617F genotyping and allele burden analysis were performed by the polymerase-chain reaction (PCR) and subsequent direct nucleotide sequencing method using the DNA of patient. For DNA analysis 73 EDTA-added peripheral blood samples and two heparinised bone marrow samples were obtained at various time points of diagnostic process. We isolated mature neutrophils from the patient blood samples as described below and used them for *JAK2* genotyping because interfusion of non-clonal lymphocytes would make the discrimination of heterozygous pattern and homozygous pattern of *JAK2*-V617F mutation rather difficult. Mature neutrophils were separated from mononuclear cells by differential centrifugation over a Ficoll-Paque PLUS (GE Healthcare Bio-sciences, Uppsala, Sweden), and pure neutrophil fraction was obtained after the removal of residual erythrocytes by 0.2% NaCl solution. DNA was extracted using the SepaGene DNA extraction Kit (Sanko Junyaku, Tokyo, Japan). *JAK2*-V617F genotyping was performed by amplifying the DNA with the following primers (Forward: 5′-AGTCATGCTGAAAGTAG GA-3′; Reverse: 5′-ATTGCTTTCCTTTTTCACA AGAT-3′) using Takara EX Taq (Takara Bio, Tokyo, Japan) and GeneAmp9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: 1 cycle at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s. PCR products were checked by 1% agarose gel with ethidium bromide, and purified using QIAquick PCR purification kit (Qiagen, Valencia, CA). Direct sequencing was performed using the Big Dye Terminator ver3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

As a human leukemic HEL cell line was confirmed to carry *JAK2*-V617F homozygous mutation, we prepared the standard samples for genotyping by mixing DNA from HEL cells and wild type DNA at various ratios. After direct sequencing of the mixed DNA samples, we measured the height of T-peak and G-peak of the 1849th nucleotide of *JAK2* gene and calculated the amount of *JAK2*-V617F mutant (T) allele divided by *JAK2* total allele (V617F mutant allele plus wild type *JAK2* allele) in percentage and regarded the value as the T allele burden (%). In this experiment, detection sensitivity of mutated DNA within total DNA was 20%. Hence, if the T allele burden was lower than 19%, the mutation pattern of the sample was regarded as the wild type. Conversely, if the T allele burden was higher than 75%, the pattern of the sample was regarded as the *JAK2*-V617F high allele burden group [*JAK2*-V617F (high)], and if the T allele burden was between 20 and 74%, the pattern of the sample as the *JAK2*-V617F middle allele burden group [*JAK2*-V617F (middle)].

JAK2-exon12 genotyping

JAK2-exon12 genotyping analysis was directed to *JAK2*-V617F-negative PV and IE patients. IE was defined as a patient group that presented varying degrees of elevated hemoglobin concentration, normal erythropoietin level and the platelet count less than 450 × 10^9/L but did not fulfill the PV criteria of the 2008 WHO criteria and showed no evidence for *JAK2* mutations. The mutational status for *JAK2*-exon12 was determined using the direct sequencing assay as previously described. The DNA was extracted from peripheral blood neutrophils of each IE patients. This genotyping was performed by amplifying the DNA with the following primers (Forward: 5′-CTCCTCTTTGGAGCAATTCA-3′; Reverse: 5′-GAGAACTTGGGAGTTGCGATA-3′) under the following conditions: one cycle at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s.

Statistical analyses

Subject characteristics were compared by Student's *t*-test or Mann–Whitney *U* test. Pearson's correlation test and nonparametric Spearman's rank order correlation tests were performed to analyse the correlations between the T allele burden and clinical/hematological parameters. *p*-Values less than 0.05 were considered significant. Statistical analyses were performed with SPSS ver.15.0.1J software.

Results

Application of 75 suspected MPN patients to the revised 2008 WHO criteria

Seventy-five patients who were diagnosed as or suspected of MPN entered this study. As the first step in this study, we diagnosed these patients based on both diagnostic criteria of the 2001 WHO classification and the revised 2008 WHO classification. As shown in Table I, 57 of 75 cases were initially diagnosed as CMPD by the 2001 criteria, whereas 59 cases were diagnosed as MPN by the 2008 criteria. This change was attributed to the two patients (one IE and one thrombocytosis patient by the 2001 criteria) who were diagnosed as ET by the 2008 criteria. However, the remaining 57 patients suspected of MPN were well accorded with both diagnostic criteria each other.

Frequency of JAK2-V617F mutation in 75 cases with MPN including suspected cases

We analysed *JAK2* status on all patients. As shown in Table II, based on the 2008 WHO classification, 59 cases were diagnosed as MPN at the first medical examination (PV 16 cases, ET 39 cases and PMF four cases). The other 16 cases, that did not fulfill the 2008 criteria and showed no evidence for *JAK2* mutations, were diagnosed as IE. The median age was 58 years old (range: 27–79 years) and the median period from the diagnosis to this entry was 60 months (range: 2–289 months). All patients were alive at the last follow-up date except one case that was diagnosed as *JAK2*-V617F-positive PV and subsequently progressed to acute myeloid leukemia.

Our method dealt with the genomic DNA from purified neutrophil fraction that contained few lymphocytes in order to precisely assess the amount of the mutant allele expression, and therefore it enabled us to distinguish whether the *JAK2*-V617F-positive patients belong to *JAK2*-V617F (high) group or *JAK2*-V617F (middle) group.

The result of the *JAK2* genotyping is also summarised in Table II. *JAK2*-V617F-positive patients were 39 cases and made up 66% of MPN patients (81% of PV, 62% of ET and 50% of PMF). Of these, *JAK2*-V617F (high) patients and *JAK2*-V617F (middle) patients were 20 and 19 cases, respectively. On contrary, all the cases with IE carried the wild type of *JAK2* gene.

Table III shows the detailed clinical/laboratory data and outcome of IE or thrombocytosis patients. Two cases were diagnosed as ET by the 2008 criteria. Hematological parameters were normalised in four cases of IE without treatment and others were in a stable state to the best of the recent information.

Clinical and laboratory features of JAK2-V617F-positive patients

Table IV shows the comparative data of clinical/laboratory findings in 59 patients with MPN classified into *JAK2*-V617F (high) group (20 cases), *JAK2*-V617F (middle) group (19 cases) and the wild type group (20 cases). WBC count, hemoglobin concentration and hematocrit value were significantly higher in *JAK2*-V617F-positive group than the wild type group. Further, WBC count, hemoglobin concentration and hematocrit value

Table I. Diagnostic alternation fo 75 suspected MPN patients by the revised diagnostic criteria of WHO classfication.

Disease phenotypes	2001 WHO classification		2008 WHO classification
Polycythemia vera	16 cases	→	16 cases
Idiopathic erythrocytosis	17 cases	→ 1 case ↓	16 cases
Essential thrombocythemia	37 cases	→	39 cases
Thrombocytosis	1 case	1 case ↑	0 case
Primary myelofibrosis	4 cases	→	4 cases

Table II. Patient characteristics and JAK2 status.

Disease phenotypes*	Case number	V617F-mutation High	V617F-mutation Middle	Wild type	V617F-positivity (%)
PV	16	11	2	3	13/16 (81)
ET	39	7	17	15	24/39 (62)
PMF	4	2	0	2	2/4 (50)
MPN total	59	20	19	20	39/59 (66)
Idiopathic erythrocytosis	16	0	0	16	0/16 (0)
Total	75	20	19	36	39/75 (52)

*Disease phenotypes were determined by 2008 WHO diagnostic criteria.

Table III. Hematological features and clinical outcome of patients with idiopathic erythrocytosis and thrombocytosis.

Patient No.	Gender	Age	WHO classification 2001	WHO classification 2008	JAK2 status	WBC ($\times 10^9$/L)	Hb (g/dl)	Hct (%)	Plt ($\times 10^9$/L)	LDH	Epo	NAP score	Spl	Karyotype	History of thrombosis	Follow up (month)	Outcome
1	M	43	IE	IE	WT	7.6	18.9	56.6	293	N	N	N	–	N	–	149	S.D.
2	M	45	IE	IE	WT	10.0	17.0	49.6	381	N	N	N	–	N	–	111	W.N.L.
3	M	58	IE	IE	WT	5.2	17.1	50.9	241	N	N	N	–	N.D.	–	109	W.N.L.
4	F	52	IE	IE	WT	8.9	19.0	61.1	222	N	N	N	–	N	AP	77	S.D.
5	M	70	IE	IE	WT	5.0	17.7	52.8	162	N	N	N	–	N.D.	–	70	W.N.L.
6	M	39	IE	IE	WT	7.9	18.3	54.1	236	N	N	N	–	N	–	60	S.D.
7	M	33	IE	IE	WT	12.8	17.2	50.5	218	N	N	N	–	N	–	53	S.D.
8	M	65	IE	IE	WT	4.2	18.4	53.7	145	N	N.D.	N	–	N.D.	–	50	W.N.L.
9	M	58	IE	IE	WT	7.4	18.1	51.6	262	N	N	N	–	45, –Y	–	42	S.D.
10	M	28	IE	IE	WT	5.0	18.2	52.8	177	N	N	N	–	N.D.	–	31	S.D.
11	M	79	IE	IE	WT	4.9	17.5	52.3	166	N	N	N	–	N.D.	–	22	S.D.
12	M	49	IE	IE	WT	9.2	20.6	59.2	232	N	N	N	–	N	–	8	S.D.
13	M	47	IE	IE	WT	6.3	20.8	59.4	241	N	N	N	–	N	AP	6	S.D.
14	F	33	IE	IE	WT	10.4	15.5	44.7	304	N	N.D.	N	–	N	–	6	S.D.
15	M	53	IE	IE	WT	9.9	16.3	48.8	310	N	N.D.	N	–	N	–	5	S.D.
16	M	61	IE	IE	WT	6.1	20.6	59.8	124	High	High	N	–	N	–	3	S.D.
17	M	56	IE	ET	WT	5.6	16.8	48.8	545	N	N	N	–	N	–	67	S.D.
18	F	65	Thromb	ET	WT	5.1	13.9	43.0	538	N	N.D.	N	–	N	–	35	S.D.

Old classification is indicative of diagnosis on the 2001 WHO CMPD classification. New classification is indicative of diagnosis on the 2008 WHO MPN classification.
Hb, Hemoglobin; Hct, Hematocrit; Plt, Platelet; LDH, lactate dehydrogenase value; Epo, erythropoietin; Spl, splenomegaly; M, male; F, female; IE, idiopathic erythrocytosis; ET, essential thrombocythemia; Thromb, Thrombocytosis; WT, wild type; N, normal; N.D., not done; AP, angina pectoris; S.D., stable disease; W.N.L., within normal range.

were significantly higher in *JAK2*-V617F (high) group than *JAK2*-V617F (middle) group. In contrast and notably, platelet count was significantly lower in *JAK2*-V617F (high) group than *JAK2*-V617F (middle) group and even lower than the wild type group. The results of statistical analyses about WBC count, hemoglobin concentration and platelet count in three groups are also shown in Figure 1.

In addition, *JAK2*-V617F (high) group had a tendency of elevated serum LDH (18 of 20 cases), elevated NAP score (all of 20 cases) and frequently revealed splenomegaly (13 of 20 cases) as compared with *JAK2*-V617F (middle) group and the wild type group. On the contrary, the incidence of the past history of thrombosis showed no significant difference among the groups.

The rate of the patients who needed continual cytoreductive chemotherapy was not significantly different among the groups.

Correlation of the mutant allele burden of JAK2 gene with disease phenotypes in JAK2-V617F-positive group

We calculated the amount of T allele expression by DNA analysis of each sample from 39 cases and analysed the correlation between the T allele burden and laboratory data. As a result, WBC count, hemoglobin concentration, hematocrit value and NAP score were positively correlated with the T allele burden ($p < 0.01$ on each factor), whereas platelet count was negatively correlated with the T allele burden ($p < 0.01$). Figure 2 shows the distribution of the T allele burden according to the disease phenotypes of 39 cases in *JAK2*-V617F-positive group (PV 13 cases, ET 24 cases and PMF two cases). The mutant allele burden of PV group was significantly higher than that of ET group ($p < 0.01$). PMF was not analysed because of a small number. This result demonstrated that the

Table IV. Clinical/laboratory findings in 59 MPN patients of various JAK2 mutation status.

Parameters	V617F-mutation High (n = 20)	V617F-mutation Middle (n = 19)	Wild type (n = 20)	Statistical evaluation
WBC count ($\times 10^9$/L)	15.06 ± 6.55	12.02 ± 4.63	8.65 ± 3.09	V617F *vs.* wild type: $p < 0.01$, High *vs.* middle: $p < 0.05$
Hemoglobin (g/dL)	17.9 ± 3.2	15.0 ± 2.4	14.7 ± 2.4	High *vs.* middle: $p < 0.01$
Hematocrit (%)	56.6 ± 9.0	46.1 ± 5.8	44.6 ± 6.6	High *vs.* middle: $p < 0.01$
Platelet count ($\times 10^9$/L)	716 ± 457	933 ± 375	781 ± 416	High *vs.* middle: $p = 0.01$
NAP score	372 ± 42	289 ± 45	218 ± 67	High *vs.* middle: $p < 0.01$
Elevated serum LDH	18/20 (90%)	11/19 (58%)	10/20 (50%)	High *vs.* middle: $p < 0.05$
Splenomegaly	13/20 (65%)	3/19 (16%)	3/20 (15%)	High *vs.* middle: $p < 0.01$
History of thrombosis	4/20 (20%)	6/19 (32%)	3/20 (15%)	High *vs.* middle *vs.* wild type: N.S.
Need for chemotherapy	13/20 (65%)	14/19 (74%)	11/20 (55%)	High *vs.* middle *vs.* wild type: N.S.

Laboratory data indicate the mean values with standard deviation, and the other data indicate the positive ratio. Statistical analysis was done between the groups as indicated, and *p*-values are shown. N.S., not significant.

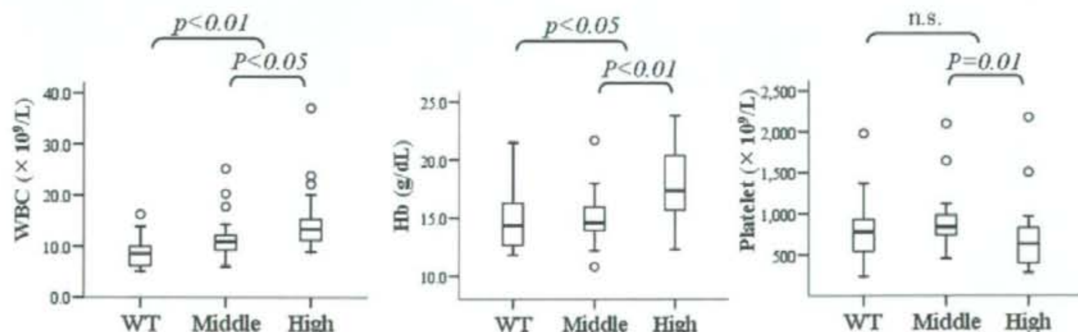

Figure 1. Hematological parameters of 59 MPN patients according to *JAK2*-V617F mutational status. WBC count (left), hemoglobin concentration (Hb; middle) and platelet count (right) between three groups are shown by box and whisker plots. WBC and platelet counts were analysed using Mann–Whitney *U* test, and hemoglobin concentration was analysed by Student *t*-test. The central box indicates the range from 25 percentiles and 75 percentiles of the measured values with the median value shown by the horizontal line in each box. *p*-Values are also indicated and n.s. means statistically not significant. Wild type group [WT]: 20 cases; *JAK2*-V617F (middle) group [Middle]: 19 cases; *JAK2*-V617F (high) group [High]: 20 cases.