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Human Flt3 Is Expressed at the Hematopoietic Stem Cell and the Granulocyte/Macrophage Progenitor Stages to Maintain Cell Survival

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FLT3/FLK2, a member of the receptor tyrosine kinase family, plays a critical role in maintenance of hematopoietic homeostasis, and the constitutively active form of the FLT3 mutation is one of the most common genetic abnormalities in acute myelogenous leukemia. In murine hematopoiesis, Flt3 is not expressed in self-renewing hematopoietic stem cells, but its expression is restricted to the multipotent and the lymphoid progenitor stages at which cells are incapable of self-renewal. We extensively analyzed the expression of Flt3 in human (h) hematopoiesis. Strikingly, in both the bone marrow and the cord blood, the human hematopoietic stem cell population capable of long-term reconstitution in xenogeneic hosts uniformly expressed Flt3. Furthermore, human Flt3 is expressed not only in early lymphoid progenitors, but also in progenitors continuously along the granulocyte/macrophage pathway, including the common myeloid progenitor and the granulocyte/macrophage progenitor. We further found that human Flt3 signaling prevents stem and progenitors from spontaneous apoptotic cell death at least through up-regulating Mcl-1, an indispensable survival factor for hematopoiesis. Thus, the distribution of Flt3 expression is considerably different in human and mouse hematopoiesis, and human Flt3 signaling might play an important role in cell survival, especially at stem and progenitor cells that are critical cellular targets for acute myelogenous leukemia treatment.


Hematopoiesis is one of the most intensely studied stem cell systems where hematopoietic stem cells (HSCs)3 self-renew, generate a variety of lineage-restricted progenitors, and continuously supply all types of mature blood cells. The technical advances of the multicolor FACS and the use of mAbs have enabled the prospective isolation of hematopoietic stem and progenitor cells according to the surface marker expression. In mice, multipotent hematopoietic activity resides in a small fraction of bone marrow (BM) cells lacking the expression of lineage-associated surface marker (Lin) but expressing high levels of Sca-1 and c-Kit (1, 2). Within the c-KitLinSCA-1+ (KLS) fraction, the most primitive self-renewing HSCs with long-term reconstituting activity (LT-HSCs) do not express murine (m) CD34, but they do express mCD38 and a low level of mCD90 (Thy1), whereas mCD34+, mCD38+, or mThy1L KLS cells are short-term HSCs (ST-HSCs) or multipotent progenitors that do not self-renew (3–5). Downstream of the mCD34+ ST-HSC stage, common lymphoid progenitors (CLPs) (6) and common myeloid progenitors (CMPs) (7) that can differentiate into all lymphoid cells and myeloid-erythroid cells, respectively, have been purified. CMPs differentiate into granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs), both of which are also prospectively isolatable by FACS (7).

Interestingly, the expression pattern of these surface markers in early stem and progenitor populations are considerably different in human (h) hematopoiesis. In humans, LT-HSCs express hCD34 (8). The hLT-HSC resides in the hCD34+hCD38– (9, 10) or the hCD34+hCD90+ (11–13) fractions in both human BM and cord blood (CB). It is still unclear what percent of hCD34+hCD38– or hCD34+hCD90+ cells are LT-HSCs in human hematopoiesis. The human counterpart for mCMPs, mGMPs, mMEPs, or mCLPs is also isolatable in the BM and the CB within the hCD34+hCD38+ progenitor fraction (14, 15). It has thus been suggested that, despite the difference in the expression patterns of key Ags in human and mouse hematopoiesis, lineage commitment processes from HSCs to mature blood cells might be generally preserved in both species. For example, the existence of prospectively isolatable CMPs and CLPs suggests that lineage commitment from HSCs involves myeloid vs lymphoid bifurcation in both mouse and human.

Recently, two independent groups have reported that in murine hematopoiesis, Flt3/Flik2, a tyrosine kinase receptor, is expressed in ST-HSCs but not in LT-HSCs. One group showed that mCD34−
KLS cells (LT-HSCs) are mFlt3− (16), and the other showed that only the mFlt3− fraction of mCD90− KLS cells possesses LT-HSC activity (17). Each group further studied the detailed differentiation activity of mFlt3+ KLS cells, but drew different conclusions. Adolfsson et al. (18) reported that the mFlt3−mCD34+ KLS population maintains the granulocyte/macrophage (GM) and the T/B lymphoid, but not the megakaryocyte/erythrocyte (MegE) potential, if any. This result suggests that, in addition to the lymphoid vs myeloid developmental pathway represented by CLPs and CMPs, respectively, there is a critical stage common to GM, T, and B lymphoid cells. The other group, however, showed that mFlt3−mCD34− KLS cells are multipotent, thus claiming that the stage common to GM/lymphoid lineages proposed by Adolfsson et al. (18) does not constitute a major pathway for hematopoietic development (19). In contrast, downstream of the mST-HSC stage, there is a general agreement that mFlt3 is expressed in progenitors with lymphoid potential, such as the majority of CLPs and a minor fraction of CMPs, that retain a weak B cell potential (20), whereas it is down-regulated in late myeloid stages, such as GMPs and MEPs (20, 21). The Flt3 ligand (FL) is required for development of CLPs from mFlt3− KLS cells, whereas mFlt3 is dispensable for HSC maintenance and myeloid development (22). These results suggest that in mouse hematopoiesis, Flt3 signaling plays an important role in lymphoid, but not in HSC or myeloid, development. The precise expression and the role of hFlt3 in human hematopoiesis, however, remain unclear. Around 40–80% of hCD34+ BM and CB cells express hFlt3 (23, 24). Although a fraction of both the hFlt3− and the hFlt3+ populations gave rise to multilineage “mixed” colonies containing all myelo-erythroid components, the hFlt3− hCD34+ and hFlt3+ hCD34− populations predominantly formed GM and erythroid colonies, respectively (23–25). It has also been shown that cells with NOD/SCID reconstitution activity reside in the hCD34− hFlt3− fraction (24). These data collectively suggest that LT-HSCs and GMPs may reside mainly in the hFlt3− hCD34− fraction, whereas MEPs may be contained in the hFlt3+ hCD34+ fraction. Therefore, the expression pattern of Flt3 could be quite different in mouse and human hematopoiesis. Flt3 expression has also been implicated in development of human acute myelogenous leukemia (AML). Flt3 is expressed in leukemic blasts in most cases with AML (26, 27). Furthermore, FLT3 is one of the most frequently mutated genes in AML (28, 29), and the FLT3 mutants transduce the constitutively active FLT3 signaling, that could be the cause of poor prognosis in AML with FLT3 mutations (30–32).

In this study, we extensively analyzed the expression and function of hFlt3 in steady-state human BM and CB hematopoiesis. Interestingly, hFlt3 was expressed in the entire human BM and CB HSC population, and purified hFlt3+ HSCs could reconstitute multilineage cells for a long-term in our xenogeneic transplantation system (33). Therefore, unlike mouse hematopoiesis, the negative expression of Flt3 does not mark LT-HSCs in human. Furthermore, in striking contrast to mouse hematopoiesis where mFlt3 is expressed in CLPs but not GMPs (20, 21), hFlt3 was expressed in GMPs as well as in CLPs at a high level. The hFlt3 signaling did not affect the lineage fate decision of hHSCs, but supported cell survival of hFlt3− stem and progenitor cells, at least through the up-regulation of Mcl-1, a survival promoting Bcl-2 homologue (34). These data collectively suggest that Flt3 signaling plays a critical role in maintenance of self-renewing LT-HSCs, and of GM and lymphoid progenitors in human hematopoiesis.

Materials and Methods

BM and CB samples

Fresh human steady-state BM and CB samples were collected from healthy adults and newborns after normal deliveries. Informed consent was obtained from all subjects. The Institutional Review Board of each institution participating in this project approved all research on human subjects.
**Figure 2.** The expression patterns of hFlt3 are similar in early human BM (A) and CB (B) hematopoiesis. In the myeloid pathway in both the BM and CB, hFlt3 was up-regulated into the GM pathway, but was down-regulated in the MegE pathway; GMPs expressed hFlt3 at a high level, whereas MEPs did not express hFlt3. CMPs contained both hFlt3⁺ and hFlt3⁻ fractions. In the lymphoid pathway, CLPs expressed hFlt3 at a high level in BM (A) and a low level in CB (B), whereas hCD10⁺ hCD19⁺ proB cells did not express hFlt3 in either the BM or the CB. Representative data of independent five experiments are shown here.

**Cell preparation, flow cytometric analysis, and cell sorting**

The BM and CB mononuclear cells were prepared by gradient centrifugation and the CD34⁺ cells were enriched from mononuclear cells by using the Indirect CD34 MicroBead kit (Miltenyi Biotec) as described previously (14). For the analyses and sorting of myeloid progenitors, cells were stained with a Cy5-PE- or PC5-conjugated lineage mixture, including anti-hCD3 (HIT3a), hCD4 (RPA-T4), hCD7, hCD8 (RPA-T8), hCD10 (H110a), hCD19 (HIB19), hCD20 (2H7), hCD11b (ICFR44), hCD14 (RMO52), hCD56 (NKH-1), and hGPA (GA-R2), FITC-conjugated anti-hCD34 (8G12), or anti-hCD45RA (HI100), PE-conjugated anti-hFlt3 (CD135) (4G8), or anti-hCD123 (6H6), allophycocyanin-conjugated anti-hCD34 (8G12), or anti-hCD38 (HIT2), Pacific Blue-conjugated anti-hCD45RA (HI100), and biotinylated anti-hCD38 (HIT2), or anti-hCD123 (9F5). The lymphoid progenitors were stained with the same lineage mixture except for the omission of anti-hCD10 and hCD19 followed by FITC-conjugated anti-hCD10 (SS2/36), PE-Cy7-conjugated anti-hCD34 (SS2/36), and anti-hFlt3, hCD34, hCD38, and hCD45RA as described above. For additional analyses, PE-Cy7-conjugated anti-hCD34 (8G12), FITC-conjugated anti-hCD90 (5E10), PE-conjugated anti-hCD117 (YB5.B8), biotinylated anti-hFlt3 (BV10A4H2), and PE-conjugated anti-hCD127 (R34.34) mAbs were used. Streptavidin-conjugated allophycocyanin-Cy7 or PE-Cy7 was used for visualization of the biotinylated Abs (BD Pharmingen). The dead cells were excluded by propidium iodide (PI) staining. Appropriate isotype-matched, irrelevant control mAbs were used to determine the level of background staining. The cells were sorted and analyzed by FACS Aria (BD Biosciences). The sorted cells were subjected to an additional round of sorting using the same gate to eliminate contaminating cells and doublets. For single-cell assays, the re-sort was performed by using an automatic cell-deposition unit system (BD Biosciences).

**In vitro assays to determine the differentiation potential of progenitors**

Clonogenic CFU assays were performed using a methylcellulose culture system that was set up to detect all possible outcomes of myeloid differentiation as reported previously (14, 35). For myeloid colony formation, cells were cultured in IMDM-based methylcellulose medium (Methocult H4100; StemCell Technologies) supplemented with 20% FCS, 1% BSA, 2 mM L-glutamine, 50 μM 2-ME, and antibiotics in the presence of human cytokines such as IL-3 (20 ng/ml), stem cell factor (SCF) (20 ng/ml), FL (20 ng/ml), IL-11 (10 ng/ml), thrombopoietin (Tpo) (50 ng/ml), erythropoietin (Epo) (4 U/ml), and GM-CSF (50 ng/ml). All cytokines were obtained from R&D Systems. Colony numbers were enumerated on day 14 of culture. For the short-term liquid cell culture, cells were cultured in IMDM with 10% FCS in the presence of cytokines described above. All of the cultures were incubated at 37°C in a humidified chamber under 5% CO₂.

**Apoptosis assay and cytokine stimulation assays in the serum-free medium**

To exclude the unexpected effects of FCS and to evaluate the effects of cytokine stimulation precisely, the cells were prepared in the FCS-free condition. The anti-apoptotic effect of FL and SCF was evaluated after 24 h.
were transplanted into irradiated (100cGy) NOD/SCID/IL2r null newborn mice. As previously reported elsewhere (37), for the reconstitution assays, the sorted cells were sorted in IMDM and then the cytokines were added. The sort of surface hFlt3 expression was determined by FACS (Fig. 2A). B, The long-term and multilineage reconstitution of human cells in mice injected with 1 x 10⁶ hFlt3+ hCD34+ hCD38+ Lin- BM cells into NOD/SCID/IL2r null newborns. Donor-derived viable human cells were evaluated as hCD45+ and hCD34+ cells. hCD34+ granulocytes, hCD14+ monocytes, hCD41+ megakaryocytes, hCD19+ B cells, hCD3+ T cells, and hCD56+ NK cells were detected in the BM of recipient mice. C, Multilineage reconstitution (upper panels) and 15 wk (lower panels) after iv. injection of 5 x 10⁶ hFlt3+ hCD34+ hCD38+ hCD90+Lin- BM HSCs into NOD/SCID/IL2r null newborns. HSCs with long-term reconstitution activity reside in the hCD34+ hCD38+ hCD90+ Lin- BM HSCs. The BM contained hFlt3+ hCD34+ hCD38+ hCD123low MEPs. The expression patterns of hFlt3 in each population were identical with those of freshly isolated stem and progenitor cells. A representative experiment by using BM samples from three independent normal donors is shown.

In vivo assays to determine the differentiation potential and reconstitution capacity

The NOD.Cg-Prkdcrexv/IL-2rgmwmWjSz (NOD/SCID/IL2rnull) mice were developed at The Jackson Laboratory. The NOD/SCID/IL2rnull strain was established by backcrossing a complete null mutation at yc locus (36) onto the NOD.Cg-Prkdcrexv strain. The establishment of this mouse line has been reported elsewhere (37). For the reconstitution assays, the sorted cells were transplanted into irradiated (100cGy) NOD/SCID/IL2rnull newborns via a facial vein within 48 h of birth. To confirm the long-term reconstitution by hHSCs, the chimerism of circulating human blood cells were analyzed until at least 24 wk after transplantation, as previously reported (33). In addition to the Abs described above, the following mAbs were used: allophycocyanin-conjugated anti-hCD45 (J33), PE-Cy7-conjugated anti-hCD123 (6H6), FITC-conjugated anti-hCD33 (HIM3-4) or hCD14 (M5E2), and PE-conjugated anti-hCD11b (VIPL3), hCD56 (B159), anti-hGlycoporphin A (GPA) (GA-R2), or anti-hCD3 (HIT3a).

Quantitative real-time PCR

To examine the gene expression profile of each population, RNA was isolated from 2,000-sorted cells using Isogen reagent (Nippon gene) according to the manufacturer’s instructions. The total RNA was reverse transcribed to cDNA using a TaKaRa RNA PCR kit (Takara Shuzo). The mRNA levels were quantified in triplicate using a real-time PCR (7500 Real-Time PCR system; Applied Biosystems). hβ2-microglobulin mRNA was separately amplified in the same plate to be used for internal control. The primer and probes were designed by Primer Express software (Applied Biosystems).

Results

The hCD34+ hCD38+ HSC fraction express hFlt3 at a low level in both BM and CB

The hCD34+ Lin- population was divided into hCD38+ and hCD38- populations (Fig. 1, A and B). It has been shown that HSCs with long-term reconstitution activity reside in the hCD38+ fraction within the hCD34+ BM and CB populations (9, 10). As shown in Fig. 1A, in the BM, hCD38+ cells constituted only ~5% of the Lin- hCD34+ population. This population uniformly expressed hFlt3 at a low level. More than...
Flt3 expression in human hematopoiesis

FIGURE 4. The lineage potential and the relationship of myeloid progenitor populations. A, Clonogenic colony formation of purified populations on methylcellulose in the presence of cytokine mixture. The hCD34+/hCD38− HSCs and hFlt3+/CMPs gave rise to various myeloid colonies including CFU-GEMM, whereas GMPs and MEPs formed exclusively GM and MegE lineage-related colonies, respectively. In contrast, hFlt3+ CMPs predominantly gave rise to MegE lineage-related colonies but failed to form CFU-GEMM. The mean value of eight independent experiments is shown. CFU-M: CFU-macrophage, CFU-G: CFU-granulocyte, CFU-GM: CFU-granulocyte/macrophage, CFU-Meg: CFU-megakaryocyte, and BFU-E: burst-forming units-erythroid. B, The lineal relationship between hFlt3+ CMPs and hFlt3+ CMPs. After 72 h of culturing, hFlt3+ CMPs gave rise to hFlt3+ CMPs, GMPs, and MEPs. In contrast, hFlt3− CMPs differentiated into only MEPs, thus suggesting hFlt3+ CMP to be a transitional intermediate population from hFlt3− CMPs to hFlt3+ MEPs. C, The colony formation activity of phenotypically defined secondary CMPs, GMPs, and MEPs purified from the primary culture of hFlt3− CMPs or hFlt3+ CMPs. Each population displayed the colony formation activity consistent with their phenotypic definition. The mean value of four independent experiments is shown.

60% of the hCD34+/hCD38− BM cells also expressed hCD90, another critical marker for hHSCs (11–13), whereas the hCD34+/hCD38−/Lin− fraction was constituted of hCD38+ lineage-committed progenitors.

In the CB, only ~30% of hCD34+/hCD38− cells expressed hCD90 (Fig. 1B). In the NOD/SCID/IL2Rγnull newborn system, the hCD34+/hCD38−/hCD90− population was highly enriched for HSCs capable of long-term reconstitution as compared with the hCD34+/hCD38−/hCD90− CB fraction (F. Ishikawa, unpublished data). The vast majority of hCD34+/hCD38− cells expressed hFlt3 at a low level as previously reported (38). Furthermore, the hCD34+/hCD38−/hCD90− CB population expressed hFlt3.

These data clearly show that hFlt3 is expressed in all cells with the hHSC phenotype in both the BM and the CB, and suggest that Flt3 expression does not discriminate ST-HSCs from LT-HSCs in human as it does in mouse (16, 17). In contrast, the BM and the CB hCD34+/hCD38− progenitor fraction expressed negative to high levels of hFlt3. We thus further subfractionated the hCD34+/hCD38− population to evaluate the hFlt3 expression in a variety of lineage-restricted progenitors.

The expression of hFlt3 within the hCD34+/hCD38− progenitor fraction

In mouse hematopoiesis, the expression of mFlt3 is associated with early lymphoid progenitor activities; it is expressed in the majority of CLPs, and in the minority of CMPs with weak B cell potential (20), but not in MEPs or GMPs (20) (21). Fig. 2 shows the expression of hFlt3 in the myeloid and lymphoid progenitor populations. According to the phenotypic definition of human myeloid and lymphoid progenitors (14, 15, 39, 40), hCD34+/hCD38− cells were subfractionated into myeloid and lymphoid progenitors, including the hCD45RA−hCD123+ (IL-3Rα)+ GMP, the hCD45RA+hCD123+ MEP, the hCD45RAhCD123low GMP, the hCD10−/hCD19− CLP, and the hCD10+/hCD19+ proB populations. Interestingly, in both the human BM and CB, ~70–80% of CMPs expressed hFlt3, whose level was progressively up-regulated at the GMP stage. In contrast, hFlt3 expression was completely shut down in MEPs. In the lymphoid lineage, the hCD34+/hCD38−/hCD10− CLP (15) strongly expressed hFlt3, whereas hFlt3 was down-regulated in the proB cells. The expression level of hFlt3 in GMPs and CLPs appears to be higher than that in hCD34+/hCD38−/hCD90− HSCs (Fig. 2). We also tested the level of hFlt3 transcripts in purified hHMSCs and progenitor populations (Fig. 3A). The pattern of hFlt3 mRNA expression was generally consistent with that in hFlt3 protein, as evaluated by using anti-hFlt3 Abs on FACS (Figs. 1 and 2). Consistent with a previous report (41), MEPs and hFlt3− CMPs had the lowest levels, GMPs and CLPs had the highest levels, and the hCD34+/hCD38− HSC population had a medium level of hFlt3 mRNA. Collectively, functional hLT-HSCs express hFlt3 mRNA...
and surface protein, and the distribution of Flt3 is quite different between human and mouse in early hematopoiesis.

In contrast, c-Kit was expressed at high levels in human HSCs and myelo-erythroid progenitors, while at a low level in CLPs (Fig. 1 C). The expression pattern of c-Kit in human hematopoietic stem and progenitor cells is generally consistent with that in mouse hematopoiesis (4, 6, 7), suggesting that the c-Kit expression program is preserved in mouse and human hematopoiesis.

hFlt3 is expressed in functional hHSCs capable of reconstituting normal hematopoiesis in the NOD/SCID/IL-2 receptor γ-chain null (NOD/SCID/IL2γnull) mouse model

In the NOD/SCID/IL2γnull newborn system, hCD34+/hCD38−/hCD90− BM and CB cells are capable of reconstitution of all hematopoietic lineages for a long term (33). The entire hCD34+/hCD38− BM population expressed hFlt3 (Fig. 1A), suggesting that functional hBM HSCs possess hFlt3 on their surface. In contrast, hCD34+/hCD38−/hCD90− CB cells contained some hCD90− cells that did not express hFlt3. To formally test whether Flt3-expressing hCD34+/hCD38− CB cells possess LT-HSC activity, we transplanted hFlt3+/hCD34+/hCD38−/hCD90− CB cells into NOD/SCID/IL2γnull newborns. As shown in Fig. 3B, NOD/SCID/IL2γnull mice transplanted with 1 × 10^6 hFlt3+/hCD34+/hCD38−/hCD90− CB cells reconstituted all hematolymphoid lineages for >6 mo, indicating that hFlt3 is expressed in functional hHSCs in CB as well as in BM.

Fig. 3C shows the phenotypic analysis of human progeny from 5 × 10^6 hFlt3+/hCD34+/hCD38−/hCD90− BM cells 6 (upper panels) or 15 wk (lower panels) after transplantation into NOD/SCID/IL2γnull newborns (33). hFlt3+/hCD34+/hCD38−/hCD90− BM cells differentiated into all hematopoietic lineage cells, including hCD33+ granulocytes, hCD14+ monocytes, hCD41+ megakaryocytes, hCD19+ B cells, hCD3+ T cells, hCD56+ NK cells (Fig. 3C), and hGPA+ erythrocytes (not shown). Furthermore, transplanted hFlt3+/hCD34+/hCD38− HSCs purified from primary recipients developed secondary hFlt3− HSCs and hFlt3−/+/MEPs, hFlt3− MEPs, and hFlt3+ GMPs recapitulating normal human hematopoietic development. Thus, the hCD34+/hCD38−/hCD90− BM population contains cells with long-term SCID reconstitution potential as reported (33, 42), and all cells within this population express hFlt3 on their surface (Fig. 3D).

The up- or down-regulation of hFlt3 in the myeloid pathway is associated with GM or MegE differentiation activity, respectively

Fig. 4A shows the differentiation potential of purified BM progenitors in vitro in the presence of the myeloid cytokine mixture containing SCF, FL, IL-3, IL-11, Tpo, Epo, and GM-CSF. hFlt3−
CMPs formed a variety of myelo-erythroid colonies including clonogenic CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM), whereas hFlt3\(^{+}\)/H11002 CMPs did not form CFU-GEMM, but preferentially differentiated into the MegE lineage. Since GMPs (hFlt3\(^{+}\)/H11001) and MEPs (hFlt3\(^{+}\)/H11002) 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\(^{+}\)/H11002 CMPs. We thus directly tested the lineage relationship of these purified myelo-erythroid progenitor populations (Fig. 4B). hFlt3\(^{+}\)/H11001 and hFlt3\(^{+}\)/H11002 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. 4C). 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. 5A, 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. 5A). 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). Without FL, a considerable proportion of purified HSCs, CMPs, and GMPs rapidly became Annexin\(^{\text{PI}}\)/PI\(^{+}\) and Annexin\(^{\text{PI}}\)/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. 6).
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. 5C, 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-xL, 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. 6A shows the distribution of the transcripts of Bcl-2 family molecules including Mcl-1, Bcl-2, and Bcl-xL 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-xL 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. 6B shows the relative expression level of Mcl-1, Bcl-2, and Bcl-xL 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-xL, 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<sup>+</sup> hCD38<sup>+</sup> hLin<sup>−</sup> 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. 6A), 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<sub>L</sub>, 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 myelo-proliferative 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 myelogenous leukemia, GMPs could be the target for blast 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 hematopoiesis. 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.

**References**


