Cbfβ Reduces Cbfβ-SMMHC–Associated Acute Myeloid Leukemia in Mice

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In preparation for the publication of this manuscript, we have synthesized the following sentence: the CBFB domain from Cbfβ was rescued in definitive hematopoiesis and died at midgestation (3–6). This phenomenon is a consequence of the fusion protein Cbfβ-SMMHC in vivo. In addition, Cbfβ-SMMHC–mediated leukemia development is accelerated in the absence of Cbfβ. These results indicate that the balance between Cbfβ and Cbfβ-SMMHC directly affects leukemia development, and suggest that CBF-specific therapeutic molecules should target CBFβ-SMMHC function while maintaining CBFβ activity. (Cancer Res 2006; 66(23): 11214-8)

Introduction

The core-binding factor (CBF) transcription factor is the most common target of chromosomal rearrangements in human acute myeloid leukemia (AML), including the fusion genes CBFB-MYH11 and RUNXI-ETO (1). Moreover, RUNX1 is frequently mutated in AML. CBF is a heterodimeric transcription factor that consists of a DNA binding α-subunit, encoded by one of three members of the RUNX family (RUNXI, RUNX2, and RUNX3), and a β-subunit encoded by the CBFB gene that increases DNA-binding affinity to the complex. In hematopoiesis, the CBF heterodimer Cbfβ/Runx1 regulates expression of genes with critical functions in differentiation of lymphoid and myeloid lineages. The Cbfβ/Runx3 complex is involved in B-cell maturation and the silencing of the CD4 gene during T-cell maturation (2). Studies in the mouse have demonstrated that Cbfβ+/− and Runx3−/− embryos fail to develop embryonic definitive hematopoiesis and die at midgestation (3–6). This phenotype was rescued in Cbfβ−/− mice expressing Cbfβ from the hematopoietic specific promoters Tie2 or GATA1, further underscoring the key role of Cbfβ during hematopoietic differentiation (7, 8).

Approximately 12% of AML patients present a chromosome 16 inversion [inv(16); ref. 9] that breaks and joins the first five exons of CBFB with the second half of the smooth muscle myosin heavy chain gene MYH11 (10). The resulting CBFB-MYH11 gene encodes the CBFβ-SMMHC fusion protein, which retains the Runx-binding domain from Cbfβ and multimerization domain from the myosin sequence. Studies in mice have shown that Cbfβ-SMMHC is a dominant inhibitor of CBF function because Cbfβ+/−MYH11 heterozygous knock-in embryos expressing the fusion protein failed to develop definitive hematopoiesis (11), as was shown for the Cbfβ- and Runx1-null embryos (3–6).

Induction of Cbfβ-SMMHC expression or Runx1-loss in adult bone marrow does not seem to affect the maintenance of long-term hematopoietic stem cells (12–14). However, Cbfβ-SMMHC expression reduces hematopoietic stem cell function by inhibiting multilineage repopulation and creating a myeloid progenitor predisposed to leukemia development (11).

Several lines of evidence suggest that Cbfβ-SMMHC may exert an incomplete block of CBF function. First, ectopic expression of the fusion protein in embryonic stem cells expressing one or both copies of Cbfβ does not inhibit differentiation in vitro (15). Second, Cbfβ+/−MYH11 knock-in hematopoietic stem cells expressing Cbfβ-SMMHC persist in the bone marrow of the chimeras (16). Third, retroviral insertional mutagenesis in Cbfβ−/−MYH11 knock-in chimeras identified common insertions in the Runx2 gene (17), suggesting that Cbfβ-SMMHC leukemic function is affected by levels of Runx proteins.

In this study, we test the hypothesis that Cbfβ modulates the Cbfβ-SMMHC effect in adult hematopoiesis and leukemogenesis. We used mice with a Cbfβ knock-out allele and a conditional Cbfβ−MYH11 knock-in allele to study adult myeloid differentiation and leukemia progression. This study provides evidence that Cbfβ modulates hematopoietic differentiation and Cbfβ-SMMHC–mediated leukemia development, and suggests that CBFβ up-regulation may efficiently counteract differentiation defects in human AML with inv(16).

Materials and Methods

The design of the conditional Cbfβ+/−MYH11 knock-in mice has previously been described (12), with the exception that monoclonal β-actin antibody (Sigma, St. Louis, MO) was used as western blot control. Expression of Cbfβ-SMMHC was induced in 3-week-old mice by activation of Cre recombinase from the Mx1Cre transgene using one to three doses of polyinosinic-polycytidylic acid (plpC) every other day (18). Heterozygous Cbfβ+/− knockout mice were generously provided by Nancy Speck (Dartmouth Medical School, Hanover, NH; ref. 4). For this study, all mice were maintained in the 129SvEv strain. In the transplantation assays, 1 × 10⁶ leukemic cells were transplanted into sublethally irradiated syngenic recipients as described elsewhere (19).

Molecular and cytology analysis. The Western blot, flow cytometry, and histopathology analyses were done as previously described (12). Fluorescence-activated cell-sorting analysis was done in peripheral blood of leukemic mice using FITC-c-kit and phycoerythrin-lineage antibodies (Lin−: B220, CD3, Gr1, and Mac1; all from BD Biosciences, San Jose, CA).

Colony forming assays. Mice with the genotypes Cbfβ+/−MYH11, Cbreff+/−MYH11, Cbfβ+/−MYH11, Cbreff+/−MYH11, Cbreff+/−MYH11, and Cbreff+/−MYH11 were injected with plpC at weaning every other day. Two days after the second injection, bone marrow cells were...
colony in vitro (12). To assess whether this effect is dependent on the presence of Cbfβ, colony-forming unit (CFU) assays were done with bone marrow cells from heterozygous floxed (Cbfβ<sup>S/M</sup>), hemizygous floxed (Cbfβ<sup>S/M</sup>), heterozygous restored (Cbfβ<sup>S/S</sup>-SMMHC), and hemizygous restored (Cbfβ<sup>S/S</sup>-SMMHC) mice (Fig. 1). The switch from Cbfβ<sup>S</sup> to Cbfβ<sup>S</sup>-SMMHC expression (switching floxed to restored allele) was induced by plpC-mediated Cre activation using the Mx1Cre transgene.

The number of myeloid and erythroid colonies was significantly reduced in hemizygous restored bone marrow progenitor cells expressing Cbfβ<sup>S</sup>-SMMHC when compared with control groups (Fig. 2A). Considering that the loss of one Cbfβ allele did not affect CFU formation (heterozygous floxed versus hemizygous floxed), these results indicate that the number of CFUs is markedly reduced at Cbfβ levels below 50%. In addition, the size of the colonies was markedly reduced in hemizygous restored progenitors when compared with controls (Fig. 2B). The Cre-lox–mediated excision in CFUs from hemizygous restored plates was confirmed by PCR analysis (data not shown). Cytology analysis of day-7 CFUs revealed the presence of all myeloid forms, with a small but consistent increase of blastlike immature cells in hemizygous restored colonies (Fig. 2C). These results revealed that the loss of Cbfβ in the context of Cbfβ-SMMHC significantly reduces the proliferation capacity of bone marrow myeloid progenitors in vitro.

**Results**

Cbfβ is essential to maintain proliferation capacity of myeloid progenitor cells expressing Cbfβ-SMMHC. We have recently shown that bone marrow cells expressing Cbfβ-SMMHC accumulate abnormal myeloid progenitors able to form myeloid colonies in vitro (12). To test whether the presence of Cbfβ<sup>S</sup> colony-forming unit (CFU) assays were done with bone marrow cells from heterozygous floxed (Cbfβ<sup>S/M</sup>), hemizygous floxed (Cbfβ<sup>S/M</sup>), heterozygous restored (Cbfβ<sup>S/S</sup>-SMMHC), and hemizygous restored (Cbfβ<sup>S/S</sup>-SMMHC) mice (Fig. 1). The switch from Cbfβ<sup>S</sup> to Cbfβ<sup>S</sup>-SMMHC expression (switching floxed to restored allele) was induced by plpC-mediated Cre activation using the Mx1Cre transgene. The number of myeloid and erythroid colonies was significantly reduced in hemizygous restored bone marrow progenitor cells expressing Cbfβ<sup>S</sup>-SMMHC when compared with control groups (Fig. 2A). Considering that the loss of one Cbfβ allele did not affect CFU formation (heterozygous floxed versus hemizygous floxed), these results indicate that the number of CFUs is markedly reduced at Cbfβ levels below 50%. In addition, the size of the colonies was markedly reduced in hemizygous restored progenitors when compared with controls (Fig. 2B). The Cre-lox–mediated excision in CFUs from hemizygous restored plates was confirmed by PCR analysis (data not shown). Cytology analysis of day-7 CFUs revealed the presence of all myeloid forms, with a small but consistent increase of blastlike immature cells in hemizygous restored colonies (Fig. 2C). These results revealed that the loss of Cbfβ in the context of Cbfβ-SMMHC significantly reduces the proliferation capacity of bone marrow myeloid progenitors in vitro.

Hemizygous bone marrow cells expressing Cbfβ-SMMHC show higher susceptibility to AML. The Cbfβ<sup>S/S</sup>-SMMHC restored mice succumb to AML with a median latency of 5 months (12). This latency is dose dependent as mice induced with three plpC doses developed AML with a median latency of 5 months, mice induced with one plpC dose had AML with a median latency of 8 months, and uninjected mice remained disease-free. To test whether the presence of Cbfβ has an effect in Cbfβ-SMMHC–mediated AML, we compared heterozygous restored and hemizygous restored mice after treatment with three doses of plpC. In the absence of Cbfβ, 100% of mice with bone marrow cells expressing Cbfβ-SMMHC developed AML with a significant

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**Figure 1.** Cbfβ alleles used in this study. Exons 1 to 6 of the Cbfβ gene are shown in boxes, and the encoded protein is shown on the right. The Cbfβ knock-out allele includes a neomycin (N) gene fused to the 3′-end of exon 5 (4). The floxed Cbfβ<sup>S/M</sup> allele includes exons 5 and 6 and a hygromycin gene (H) between loxP sites (triangle), followed by exon 5 fused to the 3′ MYH11 sequence and a neomycin gene (12). Upon Cre-mediated loxP deletion, Cbfβ-SMMHC is induced in the Cbfβ<sup>S/M</sup>-SMMHC restored allele.
acceleration of disease onset (median latency of 1.5 ± 0.5 months; P < 0.00001; Fig. 3A). Surprisingly, uninduced Mx1Cre/Cbfb+/56M mice also developed AML with similar latency to that of induced group (Fig. 3A; red dashed line, uninduced; red solid line, induced). It has previously been reported that Cre expression from the Mx1Cre transgene is leaky in mice not treated with pIpC (21). In our study, all AML samples from induced and uninduced groups exhibited deletion of the floxed sequence by PCR analysis (data not shown) and Cbfh-SMMHC expression was detected by Western blot analysis (Fig. 3A). Furthermore, secondary transplantation of Cbfb+/MYH11 AML cells derived from induced or uninduced mice produced leukemia in sublethally irradiated recipients with a median latency of 6 weeks (data not shown). These results indicate that Cbfh-SMMHC–induced AML development is accelerated in the absence of Cbfh.

**Disease pathology is dictated by Cbfh-SMMHC.** The hemizygous restored leukemic Cbfb+/MYH11 mice presented an increase in WBC count (mean, 41.7 ± 74.7 × 10⁶/mL) when compared with healthy littermates (mean, 4 × 10⁶/mL). As previously described in heterozygous restored leukemic mice, the leukemic cells from the hemizygous mice included predominant blastlike and myelomonocytic cells (Fig. 3C, top row) with the characteristic cell-surface profile c-kit+/Lin− (Fig. 3D). Disease was also marked by progressive anemia and infiltration into other organs, including the spleen and liver. Histology analysis of these organs confirmed the disruption of splenic architecture marked by infiltration of leukemic cells (Fig. 3C, middle row) and focal infiltrations of leukemic cells into the interstitial spaces of the liver (Fig. 3C, bottom row). Compromise of the thymus and lymph nodes was not observed. Taken together, the pathology of disease in hemizygous and heterozygous mice was similar, suggesting that the AML phenotype was determined by Cbfh-SMMHC.

To assess whether CBFB is frequently altered in human CBF AML samples, expression and mutation analyses of CBFB were undertaken. Sequence analysis of the CBFB coding region in a panel of 29 inv(16) AML samples identified no mutations. Expression analysis of CBFB in a panel of 285 human AML samples indicated that inv(16) AML samples had a 40% reduction in CBFB transcript when compared with CD34+ bone marrow cells (relative value, 0.4 ± 0.08), as expected by the expression of one CBFB allele. The CBFB levels in t(8;21) and non-CBF cytogenetic groups were unchanged (t(8;21) relative value, 0.9 ± 0.23; non-CBF relative value, 1.0 ± 0.32). These results indicate that the remaining CBFB allele is not frequently altered in inv(16) AMLs.

**Discussion.** Endogenous expression of Cbfβ and Cbfβ-SMMHC from the Cbfb allele of conditional knock-in mice creates a leukemia precursor that progresses to AML in a multistep process (12). Although the fusion protein is thought to act as a dominant factor in differentiation and transformation (11, 12, 16), the role of Cbfβ in
Cbfβ-SMMHC-mediated leukemia is not clear. Here we showed that the capacity of Cbfβ-SMMHC to induce AML in mice is modulated by Cbfβ.

The presence of Cbfβ is critical for embryonic definitive hematopoiesis (4) and for in vitro myeloid differentiation from Cbfβ−/− embryonic stem cells (15). Our study indicates that Cbfβ is necessary for in vitro myeloid-erythroid differentiation of bone marrow hematopoietic progenitors. In addition, because colonies were drastically reduced in the absence of Cbfβ but not in the presence of Cbfβ-SMMHC, our results support the hypothesis that Cbfβ-SMMHC may have an incomplete effect in differentiation. As Cbfβ and Cbfβ-SMMHC compete for binding with Runx proteins in bone marrow cells, basal levels of Cbfβ:Runx1 complex in hematopoietic progenitors expressing Cbfβ-SMMHC may be critical for proliferation of myeloid progenitors and delayed transformation.

Endogenous expression of Cbfβ-SMMHC and Cbfβ in bone marrow induces AML with a median latency of 5 months (12). We observed that upon Cre-lox–mediated switch from Cbfβ to Cbfβ-SMMHC expression in progenitor cells lacking a wild-type Cbfβ allele, AML latency was shortened to 6 weeks. These results strongly suggest that Cbfβ-SMMHC function is enhanced by Cbfβ loss. Surprisingly, a similar AML latency was observed between induced and uninduced groups. Probably, a small progenitor population may have undergone Cre/lox deletion due to “leaky” Cre expression from the Mx1Cre transgene (21), and thus becoming

Figure 3. Loss of Cbfβ accelerates Cbfβ-SMMHC–mediated AML. A, Kaplan-Meier survival curve of mice expressing Cbfβ-SMMHC in the presence or absence of Cbfβ. Mice induced with plpC (+) heterozygous restored [red dotted line, +/MYH11;Cre (+); n = 38], uninduced (−) heterozygous floxed [black line with star mark, +/56M (−); n = 20], uninduced hemizygous floxed [black line with circle mark, +/C0/56M (−); n = 15], untreated hemizygous restored [red dashed line, /C0/56M;Cre (−); n = 20], or treated hemizygous restored [red solid line, /C0/56M;Cre (+); n = 16]. B, Western blot analysis of Cbfβ-SMMHC and β-actin in AML samples derived from restored Cbfb mice induced (+) with plpC or uninduced (−). The Cbfb genotype of the AML cells is shown on the top. C, disease pathology analysis depicting an increase of immature leukemic cells (top row; triangle, blastlike; arrow, myeloid form; magnification, ×1,000), disruption of spleen architecture (middle row; magnification, ×100), and the presence of infiltrating leukemia cells (white arrow) in the liver (bottom row; magnification, ×100). Cells analyzed from wild-type control (left column) and leukemic mice expressing Cbfβ-SMMHC in the presence (middle column) or absence (right column) of Cbfβ. D, FACS analysis of leukemic cells from hemizygous restored mice (bottom) compared with wild type control (top) using lineage markers (Gr1, B220, Mac1, CD3) and a progenitor marker (c-kit).
a leukemia precursor. Importantly, all AML samples presented Cre-mediated deletion, suggesting that transformation is due to the Cbfβ to Cbfβ-SMMHC switch. Furthermore, the finding that CBFβ is not frequently lost in human AML argues against its role as an inv(16) cooperating tumor suppressor in AML. Rather, our results suggest that increase in the Cbfβ-cooperating tumor suppressor in AML. Rather, our results suggest that increase in the Cbfβ-cooperating tumor suppressor is unclear. However, we cannot rule out the possibility that Cbfβ loss in bone marrow could induce AML. The generation of conditional Cbfβ knock-out alleles will provide a critical tool to directly address this possibility using a genetic approach. Finally, these findings have important implications on the design of targeted therapies. One potential avenue is the identification of drugs that inhibit the fusion protein. Although candidate molecules should act to disrupt Cbfβ-SMMHC:Runx1 binding, it will be critical that CBFβ-Runx1 binding remains unaltered.

Acknowledgments

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References


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