Introduction

CCAAT/enhancer binding proteins (C/EBPs) play critical roles in myelopoiesis. Dysregulation of these proteins likely contributes to the pathogenesis of myeloid disorders characterized by a block in granulopoiesis. In one such disease, acute promyelocytic leukemia (APL), a promyelocytic leukemia–retinoic acid receptor α (PML-RARα) fusion protein is expressed as a result of a t(15;17) chromosomal translocation. Treatment of PML-RARα leukemic cells with all-trans retinoic acid (ATRA) causes them to differentiate into mature neutrophils, an effect thought to be mediated by C/EBPs. In this study, we assess the potential for cooperativity between increased C/EBP activity and ATRA therapy. We demonstrate that although both C/EBPα and C/EBPε can significantly prolong survival in a mouse model of APL, they are not functionally equivalent in this capacity. We also show that forced expression of C/EBPα or C/EBPε in combination with ATRA treatment has a synergistic effect on survival of leukemic mice compared with either therapy alone. (Blood. 2006;108:2416-2419)

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Study design

Plasmids
A rat C/EBPα cDNA (rC/EBPα) was generated by polymerase chain reaction (PCR) and cloned into the tamoxifen-inducible pBabe1am3-bb estrogen receptor (pB3pp3-hereceptor) to generate pB3pp3rC/EBPα-ER. For generation of MIG-rC/EBPα-ER, the rC/EBPα-ER fragment was excised from pB3pp3rC/EBPα-ER and cloned into the mouse stem cell virus–internal ribosomal entry site–green fluorescent protein (MSCV-IRESCFP [MIG]) retroviral vector as a BamHI-HincII fragment into the BglII-HpaI sites in MIG. Construction of MIG-hC/EBPβ-ER has been previously described. Briefly, human C/EBPβ-ER was cloned into the EcoRI-HpaI sites in MIG as an EcoRI-SacI fragment. Point mutations were made in the DNA-binding domains of rC/EBPα and hC/EBPβ at positions R289 and R211, respectively, using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Position R211 in C/EBPβ is equivalent to C/EBPαR289. The resulting mutants, C/EBPβR211A and C/EBPαR289A, were confirmed by DNA sequencing.

Cell culture
The 32Dc13 cell line was modified to express high levels of the ectopic receptor (32Dc13-eco R), thereby facilitating retroviral transduction. These cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL interleukin-3 (mIL-3)–conditioned media. BOSC23 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat-inactivated FBS, 10 ng/mL rIL-3, 10 ng/mL IL-6, and 10 ng/mL stem cell factor.

Retroviral transduction
BOSC23 cells were transfected with retroviral constructs as previously described. Retroviral supernatants were collected and used to transduce BOSC23 cells were transfected with retroviral constructs as previously described.24 Retroviral supernatants were collected and used to transduce BOSC23 cells were transfected with retroviral constructs as previously described.24 Retroviral supernatants were collected and used to transduce BOSC23 cells were transfected with retroviral constructs as previously described.24 Retroviral supernatants were collected and used to transduce BOSC23 cells were transfected with retroviral constructs as previously described.24 Retroviral supernatants were collected and used to transduce BOSC23 cells were transfected with retroviral constructs as previously described.24 Retroviral supernatants were collected and used to transduce BOSC23 cells were transfected with retroviral constructs as previously described.24

Mice
Mice were bred and maintained at the University of California at San Francisco, and their care was in accordance with Institutional Animal Care and Use Committee guidelines. To examine C/EBPs in vivo, retrovirally transduced GFP-positive leukemic no. 1111 cells (100 000 cells per well) were injected into the lateral tail vein of sublethally irradiated (4.5 Gy) 6- to 8-week-old female FVB/N mice. Each group (C/EBPα, C/EBPβ, or the mutant version of each) contained 20 mice, with 5 animals in each subgroup (placebo, 4-HT, ATRA, and 4-HT plus ATRA). Treatments (placebo, 25 mg 4-HT, and 10 mg ATRA) were administered to each subgroup as 21-day release, subcutaneous pellets (Innovative Research of America, Sarasota, FL).

Results and discussion

C/EBPα and C/EBPβ play central roles in normal myelopoiesis; therefore, it is likely that altered function of these proteins contributes to the pathogenesis of APL. In a previous study,16 we showed that expression of a tamoxifen-inducible form of C/EBPα, hC/EBPβ-ER, in leukemic cells caused them to differentiate into mature neutrophils in vivo. In the present study, we expand this approach to C/EBPα and C/EBPβ to prolong survival in a mouse model of APL. To assess the antileukemic effect of C/EBPs in this system, we transduced PML-RARα leukemic cells23 with either C/EBPα-ER or C/EBPβ-ER retrovirus and transplanted them into sublethally irradiated histocompatible mice. After leukemias developed in the recipient animals, the mice were treated with either a placebo or 4-HT to induce C/EBP activity. In mice receiving transplants with C/EBPβ-ER–transduced leukemias, treatment with 4-HT prolonged mean survival by 7 days compared with animals given a placebo (Figure 1A). Animals that received C/EBPα-ER–transduced leukemias demonstrated a more robust response following treatment with 4-HT (Figure 1B), exhibiting a mean increase in survival of 11 days compared with the placebo group. Therefore, tamoxifen-inducible forms of both C/EBPs and C/EBPβ significantly prolong survival in a mouse model of APL. Of note, 4-HT treatment of mice receiving untransduced cells had no effect on survival (data not shown).

Because C/EBPs belong to the bZIP family of DNA-binding proteins, we asked whether the ability to bind DNA was required for the antileukemic activities of C/EBPα and C/EBPβ. To address this, we mutated R289 in C/EBPα and the corresponding amino acid (R211) in C/EBPβ to alanine-generating mutants (C/EBPαR289A and C/EBPβR211A) that were devoid of DNA-binding activity (data not shown). We selected these residues for substitution based on the observation that R289 is an integral component of the protein-DNA interface in the crystal structure of a C/EBPα bZIP polypeptide bound to its cognate DNA site.25 We then transduced PML-RARα leukemic cells with C/EBPαR289A or C/EBPβR211A retrovirus and transplanted them as before. In animals receiving transplants with C/EBPβR211A-transduced leukemias, we found that treatment with 4-HT had no effect on survival (Figure 1C), thus indicating that the DNA-binding activity of C/EBPs is required for its antileukemic effect. We observed a similar phenomenon in the factor-dependent myeloid progenitor cell line 32Dc13 (Figure 1D). While the wild-type C/EBPα suppressed growth of 32Dc13 cells in the presence of 4-HT, the R211A mutant was unable to repress proliferation, suggesting that the DNA-binding activity of C/EBPα is required for its function in immature myeloid cells.

Interestingly, mice receiving transplants with C/EBPαR289A-transduced leukemias exhibited a mean increase in survival of 8 days compared with the placebo group following treatment with 4-HT (Figure 1E). This finding indicates that, unlike C/EBPα, C/EBPβ can suppress leukemia without binding DNA. 32Dc13 cells transduced with either wild-type or mutant C/EBPα fail to proliferate following treatment with 4-HT, suggesting that C/EBPα has antiproliferative effects that do not require DNA binding (Figure 1F). Together, the results presented in Figure 1 imply that C/EBPα and C/EBPβ are not functionally equivalent in their ability to suppress growth and inhibit leukemogenesis.

The results shown in Figure 1, in conjunction with the idea that C/EBPs mediate the antileukemic effects of ATRA, led us to assess...
the potential for cooperativity between treatment with ATRA and forced expression of C/EBPs. To address this relationship, we transduced PML-RAR<sup>H9251</sup> leukemic cells with either C/EBP<sup>H9251</sup>-ER or C/EBP<sup>H9280</sup>-ER retrovirus and transplanted them as before. After leukemias developed in recipient animals, 5 mice from each group were given either a placebo or 25 mg 4-HT. Animals that received C/EBP<sup>H9251</sup>-ER–transduced leukemias exhibited prolonged survival following treatment with 4-HT (mean survival time of 33 days compared with 26.4 days in the placebo group; $P = .002$). C/EBP<sup>R211A-ER</sup> had no effect on survival of leukemic mice (mean survival time of 37.4 days compared with 36.4 days in the placebo group). In animals receiving transplants with C/EBP<sup>H9280</sup>-ER– or C/EBP<sup>R289A-ER</sup>–transduced leukemias, treatment with 4-HT significantly prolonged survival ($P = .003$). Mean survival times for placebo and 4-HT–treated groups were 33.8 days and 46.4 days, respectively, for C/EBP<sup>H9280</sup>-ER mice and 31.4 days and 39.8 days, respectively, for C/EBP<sup>R289A-ER</sup> mice.

Animals that received C/EBP<sup>H9251</sup>-ER–transduced leukemias demonstrated a similar pattern of response; however, the magnitude of each response was greater than that seen in C/EBP<sup>H9280</sup>-ER mice (Figure 1B). Treatment with 4-HT and ATRA prolonged the survival of C/EBP<sup>H9280</sup>-ER animals by a mean increase of 11 and 38 days, respectively, but the greatest impact on survival was seen in mice given the combined treatment ($P < .001$). Mean survival times of placebo, 4-HT, ATRA, and 4-HT plus ATRA groups were 26.4, 33, 49.6, and 63 days, respectively, in animals receiving transplants with C/EBP<sup>H9280</sup>-ER–transduced leukemias and 30.5, 41.8, 69, and 97.8 days, respectively, in C/EBP<sup>H9251</sup>-ER animals.


7. Landschulz WH, Johnson PF, McKnight SL. The DNA binding domain of the rat liver nuclear protein C/EBP is bipartite. Science. 1989;243:1681-1688.


CCAAT/enhancer binding proteins alpha and epsilon cooperate with all-trans retinoic acid in therapy but differ in their antileukemic activities

Young-Jin Lee, Letetia C. Jones, Nikolai A. Timchenko, Danilo Perrotti, Daniel G. Tenen and Scott C. Kogan