Increased CCAAT Enhancer-binding Protein ε (C/EBPε) Expression and Premature Apoptosis in Myeloid Cells Expressing Gfi-1 N382S Mutant Associated with Severe Congenital Neutropenia*

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Granulocyte-colony-stimulating factor (G-CSF) stimulates the activation of multiple signaling pathways, leading to alterations in the activities of transcription factors. Gfi-1 is a zinc finger transcriptional repressor that is required for granulopoiesis. How Gfi-1 acts in myeloid cells is poorly understood. We show here that the expression of Gfi-1 was up-regulated during G-CSF-induced granulocytic differentiation in myeloid 32D cells. Truncation of the carboxyl terminus of the G-CSF receptor, as seen in patients with acute myeloid leukemia evolving from severe congenital neutropenia, disrupted Gfi-1 up-regulation by G-CSF. Ectopic expression of a dominant negative Gfi-1 mutant, N382S, which was associated with severe congenital neutropenia, resulted in premature apoptosis and reduced proliferation of cells induced to differentiate with G-CSF. The expression of neutrophil elastase (NE) and CCAAT enhancer-binding protein ε (C/EBPε) was significantly increased in 32D cells expressing N382S. In contrast, overexpression of wild type Gfi-1 abolished G-CSF-induced up-regulation of C/EBPε but had no apparent effect on NE up-regulation by G-CSF. Notably, G-CSF-dependent proliferation and survival were inhibited upon overexpression of C/EBPε but not NE. These data indicate that Gfi-1 down-regulates C/EBPε expression and suggest that increased expression of C/EBPε as a consequence of loss of Gfi-1 function may be deleterious to the proliferation and survival of early myeloid cells.

The production of mature granulocytes, a process known as granulopoiesis, is regulated, at least in part, by a network of hematopoietic cytokines and growth factors. Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic cytokine that plays a major role in granulopoiesis (1). G-CSF supports the proliferation, differentiation, and survival of myeloid progenitor cells. The biological activities of G-CSF are mediated by its cognate receptor, a member of the cytokine receptor superfamily (2, 3). Treatment of cells with G-CSF activates multiple intracellular signal transduction pathways leading to up- or down-regulation of genes that either positively or negatively regulate myeloid development.

Regulation of gene expression in myeloid cells is achieved by coordinated activities of transcriptional activators and repressors. Transcription factors including the CCAAT enhancer-binding protein α (C/EBPα), C/EBPε, PU.1, and c-Myb have been implicated in myeloid development. Expression of some of the myeloid transcription factors such as C/EBPε and C/EBPα is regulated by G-CSF during granulocytic differentiation (4–6). Notably, mutations in the genes encoding C/EBPε and PU.1 are associated with acute myeloid leukemia (AML) (7–9). The roles of transcriptional repressors in granulopoiesis are poorly understood, and it remains largely unexplored as to the expression and function of myeloid transcriptional repressors during granulocytic development.

Gfi-1 encodes a nuclear zinc finger transcriptional repressor that is expressed in hematopoietic system (10). Gfi-1 was first identified as a target gene in a retroviral insertion screen for T cell interleukin-2-independent growth (11). Subsequent studies showed that Gfi-1 promoted proliferation and inhibited apoptosis in T cells (12–15). Recent studies further implicated Gfi-1 as an important regulator of stem cell self-renewal ability (16, 17). Surprisingly, mice with targeted disruption of Gfi-1 not only showed defective T cell development but also were severely neutropenic (18, 19). Bone marrow cells from Gfi-1 knock-out mice failed to produce mature granulocytes in response to G-CSF in colony formation assays. These studies indicated that Gfi-1 plays a critical role in granulopoiesis.

Severe congenital neutropenia, also called Kostmann syndrome, is characterized by early onset of bacterial infections resulting from severe absolute neutropenia and a differentiation block of marrow myeloid cells at early stages of development. Mutations in ELA2, which encodes a myeloid-specific serine protease neutrophil elastase (NE), have been identified in more than 50% of patients with SCN (20–22). Although not very common, mutations in GFI-1 have been reported in neutropenic patients with no ELA2 mutations (23). Notably, ~10% of patients with SCN eventually develop AML, and with rare exceptions, these AML patients carry mutations in the CSF3R encoding the G-CSF receptor (24–28). The mutations cause truncation of the carboxyl-terminal region of the G-CSF receptor, a region that negatively regulates G-CSF-stimulated proliferation signaling and is involved in induction of granulocytic differentiation in both myeloid cell lines and transgenic mice (24, 29, 30). When expressed in murine hematopoietic cells, the truncated G-CSF receptors derived from AML/SCN patients mediate augmented and prolonged activation of Stat5, Akt, and Erk1/2 pathways (31–34).

Despite its critical role in granulopoiesis, how Gfi-1 functions in myeloid cells remains poorly understood. In this study, we show that Gfi-1 expression was up-regulated during G-CSF-induced granulocytic dif-
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Cell differentiation and that the carboxyl-terminal region of the G-CSF receptor was required for Gfi-1 up-regulation by G-CSF. We also show that expression of a dominant negative Gfi-1 mutant, associated with SCN, suppressed the proliferation and survival of cells stimulated by G-CSF and led to increased expression of NE and C/EBPε. We further demonstrate that overexpression of C/EBPε, but not NE, attenuated G-CSF-dependent cell proliferation and survival. These data indicate that Gfi-1 represses C/EBPε expression, and a potential role of Gfi-1 in granulopoiesis is to prevent C/EBPε overexpression at early stages of granulocytic differentiation.

EXPERIMENTAL PROCEDURES

Cell—Murine 32D cells stably transfected with the wild type and/or the truncated forms of the human G-CSF receptor have been described (24, 34). The 32D cells used in this study did not express the endogenous G-CSF receptor. L-G cells (35) were kindly provided by Dr. T. Honjo (Kyoto University). 32D and L-G cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10% WEHI-3B cell conditioned medium as a crude source of interleukin-3 (IL-3), 100 μg/ml penicillin, and 100 μg/ml streptomycin.

Reagents—Antibodies against Gfi-1, NE, C/EBPα, C/EBPε, and PU.1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Bak and Bax were obtained from Oncogene Research Products (San Diego, CA). Anti Bcl-2 and Bcl-X, antibodies were purchased from BD Biosciences. Anti β-actin antibody was from Sigma. [α-32P]dCTP and ECL kit were purchased from PerkinElmer Life Sciences and Pierce, respectively.

Expression Constructs and Transfection—The retroviral Gfi-1 expression construct Gfi-1-RV containing an internal ribosomal entry sequence (IRES) and humanized GFP-cDNA (14) was kindly provided by Dr. J. Zhu (National Institutes of Health). Gfi-1 N382S mutant (N382S-RV) was generated by site-directed mutagenesis. The mutation created a XhoI site in Gfi-1 cDNA, and the presence of the mutation was confirmed by digestion with XhoI. The cDNA encoding human NE was kindly provided by Dr. M. Horwitz (University of Washington School of Medicine) and cloned into retroviral expression vector pBage Puro. The C/EBPε expression construct mouse stem cell virus-human C/EBPε-32-IRES-GFP has been described (36). 32D cells were transfected by electroporation with the different expression constructs, together with 32-IRES-GFP has been described (36). 32D cells were transfected by electroporation with the different expression constructs, together with

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Analysis of DNA Fragmentation—Genomic DNA was extracted from cells essentially as described (37). Cells (4 × 10⁶) were washed and incubated overnight at 42 °C in 400 μl of digestion buffer containing 10 mM Tris (pH 7.5), 400 mM NaCl, 2 mM EDTA, 1% SDS, and 1.5 mg/ml proteinase K. 130 μl of 6 mM NaCl was added and centrifuged at 8,000 rpm for 15 min after vigorous shaking. The supernatants were collected and mixed with 2 volumes of absolute ethanol prior to centrifugation at 12,000 rpm for 30 min. DNA pellets were washed in 70% ethanol, air-dried, and dissolved in TE buffer (1 mM NaCl, 0.1 mM EDTA).

Cell Cycle Analysis—Cells were collected at different times of G-CSF treatment and fixed with 70% ethanol at −20 °C prior to staining with propidium iodide (50 μg/ml) in the presence of 0.05% Triton X-100, 100 μg/ml RNase A, and 1% bovine serum albumin. Flow cytometry was performed on a FACScalibur (Becton Dickinson), and the data were analyzed using the CellQuest program.

RESULTS

Because Gfi-1 is required for granulopoiesis, we examined the expression of Gfi-1 at the different stages of G-CSF treatment in myeloid 32D cells stably transfected with the wild type (WT) G-CSF receptor and/or the d715 mutant associated with SCN/AML (24–28) (Fig. 1A). The 32D cells used in this study expressed no endogenous G-CSF receptor. The expression of the transfected receptor proteins was confirmed by Western blot analysis (Fig. 1B) and flow cytometric analysis (data not shown). As previously reported (29, 38), the d715 receptor was expressed more abundantly than the WT receptor. 32D cells expressing the WT G-CSF receptor (32D/WT) proliferated transiently and terminally differenti-
ated into mature granulocytes upon induction with G-CSF. In contrast, 32D cells expressing the d715 receptor (32D/d715) or both the WT and the d715 receptors (32D/WT/d715) grew continuously but failed to differentiate in G-CSF (24, 29, 39). The different 32D cells were cultured in G-CSF for various times, after which RNA was extracted and examined for Gfi-1 expression by Northern blot analysis. Gfi-1 mRNA increased steadily in 32D/WT cells, reaching peak level by day 6 and declining thereafter (Fig. 1C). No significant up-regulation of Gfi-1 mRNA was observed when 32D/d715 cells were cultured in G-CSF for up to 8 days. Gfi-1 up-regulation by G-CSF was markedly attenuated in 32D/WT/d715 cells. These data indicate that the carboxyl-terminal region of the G-CSF receptor was required for induction of Gfi-1 by G-CSF and that the d715 mutant exerted a dominant negative effect on Gfi-1 up-regulation mediated by the wild type G-CSF receptor.

Mutations in GFI-1 gene have been reported in neutropenic patients (23). The Gfi-1 mutants derived from the patients acted in a dominant negative manner. To evaluate the importance of Gfi-1 function in cellular response to G-CSF, we transfected 32D/WT cells with Gfi-1 or the N382S mutant, which was associated with SCN (23). The expression of Gfi-1 and the N382S mutant was confirmed by Western blot analysis (Fig. 2A). As compared with cells transfected with the empty vector (32D/Puro) or Gfi-1 (32D/Gfi-1), 32D/WT cells expressing the N382S mutant (32D/N382S) grew somewhat slower but showed comparable viability in IL-3-containing culture medium (Fig. 2B and data not shown). Upon transfer to medium containing G-CSF, 32D/N382S cells displayed markedly reduced growth and rapidly lost viability (Fig. 2C and D). The inhibitory effect of the N382S mutant on cell proliferation and survival was seen in six independent 32D/N382S clones including those expressing low levels of N382S protein (data not shown). 32D/Puro and 32D/Gfi-1 cells displayed comparable growth and survival in culture medium containing IL-3 or G-CSF. Unlike T cells that became growth factor-independent upon Gfi-1 overexpression (12–15), 32D/
Gfi-1 cells were fully dependent on IL-3 for proliferation and survival (data not shown).

We assessed whether expression of the N382S mutant affected cell cycle distributions by measuring DNA content after propidium iodide staining. 32D/N382S cells exhibited increased G0/G1 and decreased S/G2 distributions as compared with 32D/Puro and 32D/Gfi-1 cells (Fig. 3). Although 32D/N382S cells died rapidly in G-CSF, treatment with G-CSF for up to 2 days did not significantly alter their cell cycle distributions. Morphological examination of 32D/N382S cells cultured in G-CSF for 3 days revealed that a large proportion of cells displayed the features characteristic of apoptosis including membrane blebbing, shrinkage of cytoplasm, and condensation/fragmentation of nuclei but not terminal granulocytic differentiation (Fig. 4A). Consistent with apoptotic morphological features, genomic DNA isolated from G-CSF-treated 32D/N382S generated a typical oligonucleosomal DNA ladder (Fig. 4B). Notably, although no significant cell death was observed when 32D/N382S cells were cultured in IL-3, DNA from the cells consistently produced a ladder pattern. 32D/Puro and 32D/Gfi-1 cells remained morphologically immature and showed no significant cell death at day 3 of G-CSF treatment (Fig. 4A). At day 9 of G-CSF induction, most 32D/Puro cells differentiated into mature granulocytes with segmented nuclei. As compared with 32D/Puro cells, 32D/Gfi-1 cells appeared morphologically less mature after G-CSF treatment for 9 days.

Gfi-1 has been shown to repress the expression of the proapoptotic Bcl-2 family members Bax and Bak, thereby inhibiting T cell death induced by IL-2 withdrawal (40). We investigated the possibility that the N382S mutant caused apoptosis by up-regulating the expression of Bax and Bak. Whole cell extracts were prepared from 32D/Puro, 32D/Gfi-1, and 32D/N382S cells maintained in IL-3 or incubated in G-CSF for different days. The levels of Bax and Bak were examined by Western blot analysis. Expression of N382S mutant did not significantly increase the levels of Bax and Bak in 32D cells (Fig. 5). We also examined the expression of the antiapoptotic Bcl-2 family members Bcl-2 and Bcl-XL. As shown in Fig. 5, the levels of Bcl-2 and Bcl-XL were not significantly decreased in 32D/N382S cells.

The ELA2 gene encoding NE has been identified as a target of Gfi-1 (19, 23, 41). NE expression in the different 32D clones was examined by Northern blot analysis. NE mRNA was low in 32D cells cultured in IL-3 and induced upon G-CSF treatment (Fig. 6A). Induction of NE expression by G-CSF was comparable in 32D/Puro and 32D/Gfi-1 cells but was significantly greater in 32D/N382S cells. In addition to ELA2, the genes encoding several myeloid transcription factors have been shown to contain Gfi-1 binding sites and may represent potential Gfi-1 targets (41). We examined the expression of C/EBPα/H9251, C/EBPβ/H9280, and PU.1, which are critically implicated in myeloid development. The protein levels of C/EBPα and PU.1, which are critically implicated in myeloid development. The levels of C/EBPα and PU.1 were comparable in the different 32D clones (Fig. 6B). In contrast, the amount of C/EBPβ was significantly higher in 32D/N382S cells than in 32D/Puro and 32D/Gfi-1 cells. In line with previous reports (5, 6, 42), G-CSF treatment resulted in dramatic increase in the amount of C/EBPβ in 32D/Puro cells (Fig. 6C). However, C/EBPβ up-regulation was completely blocked in 32D/Gfi-1 cells. Together, these data indicated that Gfi-1 repressed the expression of C/EBPβ in 32D cells.

To investigate whether expression of the N382S mutant in other myeloid cells may have similar effects on C/EBPβ expression and cellular response to G-CSF, we stably transfected murine myeloid L-G cells with the N382S mutant (Fig. 7A). L-G cells expressed the endogenous G-CSF receptor and terminally differentiated into mature granulocytes...
in response to G-CSF (35). Expression of the N382S mutant in L-G cells dramatically increased the level of C/EBPβ but did not affect the expression of C/EBPα and PU.1. Similar to 32D/N382S cells, L-G cells transfected with the N382S mutant died rapidly upon transfer to G-CSF-containing medium (Fig. 7B). Thus, the N382S mutant augmented C/EBPβ expression and suppressed G-CSF-stimulated cell proliferation and survival in both 32D and L-G cells.

To explore whether increased expression of NE and C/EBPβ contributed to the negative effect of the N382S mutant on cell proliferation and survival, we overexpressed NE and C/EBPβ in 32D/WT cells (Fig. 8A). 32D/WT cells overexpressing NE and C/EBPβ were cultured in G-CSF for different days. As shown in Fig. 8B, overexpression of NE had no apparent effect on G-CSF-dependent proliferation and survival. In contrast, overexpression of C/EBPβ markedly inhibited the proliferation and survival of 32D cells cultured in G-CSF. Similar to 32D/N382S cells, 32D/WT cells overexpressing C/EBPβ (32D/CEBPβ) displayed typical morphological features of apoptosis but not terminal granulocytic differentiation (data not shown). C/EBPβ overexpression had no significant effect on the proliferation and survival of 32D/WT cultured in IL-3 (data not shown).

**DISCUSSION**

Targeted gene disruption in mice has revealed a critical role of Gfi-1 in granulopoiesis (18, 19). However, the mechanism by which Gfi-1 functions in myeloid cells remains obscure. In this study, we have shown that Gfi-1 mRNA is up-regulated during granulocytic differentiation induced by G-CSF. We have also shown that the cytoplasmic domain of the G-CSF receptor, truncated in patients with AML/SCN, is required for Gfi-1 up-regulation by G-CSF. Our data further demonstrate that expression of the N382S mutant of Gfi-1, associated with SCN, causes premature apoptosis and reduced proliferation of myeloid 32D and L-G cells induced to differentiate with G-CSF. Together, these results indicate that Gfi-1 function is required for the survival and proliferation of myeloid cells and may explain why loss of Gfi-1 function, as a result of either gene knock-out in mice or gene mutations in humans, results in a block of granulocytic differentiation and severe neutropenia.

Gfi-1 represses transcription by binding to specific DNA sequences in target genes (43). The N382S mutant is defective in DNA binding and acts in a dominant negative manner to block repression by Gfi-1 (23). ELA2 encoding NE has been identified as the Gfi-1-target gene. Expression of NE was elevated in hematopoietic cells from patients with N382S mutation and Gfi-1-deficient mice (19, 23). Consistent with these studies, expression of the N382S mutant in 32D cells resulted in a greater increase in NE transcript in response to G-CSF. Notably, mutations in ELA2 are present in the majority of patients with SCN, and the NE mutants derived from SCN patients cause premature apoptosis of differentiating myeloid cells (27, 44). It has been suggested that Gfi-1 mutants may achieve a similar effect as NE mutants by causing NE overexpression (22). However, overexpression of NE failed to significantly influence G-CSF-dependent proliferation and survival in myeloid 32D cells. Our data argue against NE overexpression as a primary mechanism by which the N382S mutant exerts its negative effect on granulocytic differentiation, although the possibility cannot be excluded that NE overexpression may play a contributing role in the pathogenesis of neutropenia.

In addition to NE, expression of the N382S mutant in myeloid 32D and L-G cells dramatically increased the level of C/EBPβ, whereas the levels of C/EBPα and PU.1 were not significantly altered. Furthermore,
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overexpression of Gfi-1 in 32D cells completely abolished G-CSF-induced up-regulation of C/EBPε but had no apparent effect on NE up-regulation by G-CSF, suggesting that Cebpe, which encodes C/EBPε, is more sensitive to repression by Gfi-1 than Ela2. Our results are in contrast to a recent report showing that C/EBPε transcript was decreased in the bone marrow cells from Gfi-1-deficient mice (19). However, Gfi-1-deficient mice lack mature granulocytes in the bone marrow, whereas the majority of granulocytes in the bone marrow of normal mice are mature at early stages of differentiation. Because C/EBPε expression increases dramatically with terminal granulocytic differentiation (6, 45, 46), the lack of mature granulocytes in Gfi-1-deficient mice may explain why C/EBPε expression was reduced in Gfi-1-deficient bone marrow cells. In support of this notion, C/EBPε level was lower in 32D/N382S cells than in 32D/Puro cells treated with G-CSF for 6 days (data not shown).

It should be pointed out that up-regulation of Gfi-1 during G-CSF-induced granulocytic differentiation appears contradictory to the markedly increased level of C/EBPε in late granulocytes. However, Gfi-1 binding to the promoter of CEBPE diminishes sharply with granulocytic differentiation (41), presumably as a result of more closed chromatin configuration in late granulocytes. Recruitment of Gfi-1 to other potential target genes also decreases during the course of granulocytic differentiation (41). Thus, it is possible that Gfi-1 up-regulation may represent a compensatory mechanism for the loss of Gfi-1 binding to its target genes in differentiating granulocytes.

Overexpression of C/EBPε in myeloid cells has been shown to suppress cell growth and survival and drive granulocytic differentiation (6, 36, 46). In agreement with these studies, overexpression of C/EBPε in immature 32D/WT cells inhibited G-CSF-dependent proliferation and survival. 32D/WT cells overexpressing C/EBPε showed morphological features of apoptosis when cultured in G-CSF, with no apparent evidence of terminal granulocytic differentiation. It is possible that although C/EBPε is highly expressed in late granulocytes and is required for terminal granulocytic differentiation (8), overexpression of C/EBPε in immature myeloid cells may have an adverse effect on granulocytic differentiation by causing accelerated apoptosis of differentiating cells. Thus, our data suggest that a key function of Gfi-1 in granulopoiesis, among others, is to control the expression of C/EBPε and that loss of Gfi-1 function may lead to overt overexpression of C/EBPε at early stages of granulocytic development, which may contribute to the premature apoptosis of differentiating myeloid cells.

Despite its critical involvement in regulating the proliferation and survival of myeloid cells, whether Gfi-1 plays an active role in granulocytic differentiation remains to be determined. Our data show that the carboxyl terminus of the G-CSF receptor, involved in granulocytic differentiation, is required for Gfi-1 up-regulation by G-CSF. However, overexpression of Gfi-1 in 32D cells does not appear to have a positive effect on granulocytic differentiation induced by G-CSF. In fact, 32D/Gfi-1 cells appear to be morphologically less mature than 32D/Puro cells, which may be attributable, at least in part, to the dramatic suppression of C/EBPε expression. These results suggest that Gfi-1 may not function to directly drive granulocytic differentiation. Further studies are needed to address whether Gfi-1 up-regulation is required for terminal granulocytic differentiation in response to G-CSF.

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