RAPID COMMUNICATION

Suppression of Apoptosis During Cytokine Deprivation of 32D Cells Is Not Sufficient To Induce Complete Granulocytic Differentiation

By Jill E. Rodel and Daniel C. Link

The role of cytokines in the control of hematopoietic cell differentiation remains controversial. Two general models for the cytokine control of hematopoietic differentiation have been proposed. In the stochastic model, cytokines provide proliferative and survival signals to the differentiating hematopoietic cell, but they do not provide specific lineage commitment signals. In the instructive model, cytokines transmit specific signals to multipotent hematopoietic cells, thereby directing lineage commitment. To distinguish between these two models with respect to granulocyte colony-stimulating factor (G-CSF) and granulocytic differentiation, we used the 32Dc13 cell line, which is capable of differentiating into granulocytes in response to G-CSF. 32D cells transfected with either bcl-2 or bcl-X, showed prolonged survival in medium containing no cytokine supplement. Cells surviving in these cultures developed the segmented nuclei characteristic of mature neutrophils. However, no induction of myeloperoxidase activity or increase in cathepsin G transcripts were detected. These data support a hybrid model for the role of G-CSF in granulocytic differentiation; although some features of granulocytic differentiation, namely nuclear segmentation, do not require G-CSF and appear therefore to be preprogrammed in 32D cells, the complete maturation of these cells to granulocytes appears to be dependent on G-CSF.

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T HE GRANULOCYTE colony-stimulating factor (G-CSF) is a polypeptide growth factor that regulates the production, differentiation, and function of neutrophilic granulocytes.1 Its effects are mediated through its interaction with the G-CSF receptor (G-CSFR), a member of the cytokine receptor superfamily.1 The importance of G-CSF to granulopoiesis was recently shown in mice carrying the hypomorphic null mutation for G-CSF.2 These mice had approximately 20% of normal circulating neutrophils and a corresponding decrease in myeloid precursors in their bone marrow (BM).2 The mechanism by which G-CSF, and hematopoietic growth factors in general, regulate hematopoiesis is controversial. Two general models for the role of cytokines in controlling hematopoietic differentiation have been proposed.3 In the instructive model,4 cytokines transmit specific signals to multipotent hematopoietic cells directing lineage commitment. In the stochastic model,5-7 cytokines support the proliferation and survival of lineage committed cells. A major distinction between these two models is that in the instructive model the cytokine receptors are transmitting specific lineage commitment signals.

Two recent reports highlight the controversy with respect to G-CSF. Fairbairn et al5 showed that constitutive expression of the oncprotein bcl-2 in FDCP-Mix cells (a multipotent interleukin-3 [IL-3]--dependent hematopoietic cell line) delayed apoptosis upon IL-3 removal. Examination of the cells surviving in these cultures showed multilineage hematopoietic differentiation, including granulocytic differentiation. These data support a hybrid model for the role of G-CSF in granulocytic differentiation that is independent of hematopoietic growth factors. On the other hand, Dong et al9 recently identified point mutations in the G-CSFR of two patients with Kostmann syndrome who developed acute myeloid leukemia. Kostmann syndrome is a rare congenital disorder manifested by neutropenia and an arrest of myeloid maturation at the promyelocyte or myelocyte stage.10,11 The point mutations caused a truncation of the carboxy-terminal cytoplasmic region of the G-CSFR. Expression of the truncated receptor in a myeloid cell line yielded cells that proliferated rather than differentiated in response to G-CSF. These data suggest that the carboxy-terminal region of the G-CSFR may be transmitting specific differentiation signals. In agreement with these data, several recent reports have identified a putative differentiation domain in the carboxy-terminal region of the G-CSFR.12,13

We have chosen to examine the role of G-CSF in granulocytic differentiation by using the 32Dc13 model of in vitro myeloid differentiation. 32Dc13 (here simply designated 32D) cells are a nontumorigenic, diploid cell line that proliferates indefinitely in the presence of IL-3.14 In the absence of IL-3 and in the presence of G-CSF, these cells undergo granulocytic differentiation. We generated stably transfected 32D clones that constitutively express either bcl-2 or bcl-X, and examined their survival and morphology in cultures without IL-3. We show that these cells have a prolonged survival in such cultures, but do not undergo complete granulocytic differentiation without G-CSF.

MATERIALS AND METHODS

Cells and cell culture. 32Dc13 cells were provided by Dr James N. Ihle (St Jude Children’s Research Hospital, Memphis, TN) and were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 15% WEHI conditioned medium as a source of IL-3, 10% fetal bovine serum (Hartland, Indianapolis, IN), and L-glutamine (CM + IL-3). WEHI cells were provided by Dr Greg Longmore (Washington University, St Louis, MO) and were main-
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Fig 1. bcl-2 or bcl-XL expression in transfected 32D clones. bcl-2 (lanes 1 and 2) or bcl-XL (lanes 3 and 4) immunoblots of representative clones. The bcl-2 antiserum is specific for human bcl-2 and therefore does not detect endogenous murine bcl-2. Blots were stripped and incubated with an anti-β-tubulin antibody to control for protein loading.

DNA constructs and transfections. The eukaryotic expression vector used, pBSRaEN (a gift of Dr Andrey Shaw, Washington University, St Louis, MO), produces a single bicistronic message encoding the protein of interest and the neomycin phosphotransferase gene transcribed from the SRα promoter.15 cDNAs encoding human bcl-218 or murine bcl-XL (unpublished sequence) were subcloned into pBSRaEN (both cDNAs were gifts of Dr Stanley Korsmeyer, Washington University, St Louis, MO).

32D cells were transfected by electroporation. Cells, 5 x 10⁶ per sample, were washed in RPMI medium and resuspended in 300 µL of RPMI. Twenty-five micrograms of pBSRaEN vector and constructs containing bcl-2 or bcl-XL, were mixed with the cells and transferred to 2-mm gap cuvettes (BTX, San Diego, CA) on ice. Electroporation was performed using a BTX 6000 electroporation device at 200 V, 1,200 µF capacitance, and a resistance setting of R4. Samples were transferred to CM + IL-3 and incubated at 37°C. After 24 hours the cells were washed and resuspended in CM + IL-3 with G418 at a concentration of 0.8 mg/mL (GIBCO). Clonal stable transfectants were selected by limiting dilution and culture in 96-well microtiter plates.

Antisera and immunoblotting. Cells were washed once with RPMI and lysed in Tris-buffered saline (TBS) containing 1% NP40,
the number of viable cells per milliliter by the initial cell concentration. Data are shown as the mean value ± SD of two independent experiments.

1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.1 U/mL aprotinin, and 10 μg/mL leupeptin and incubated at 4°C for 10 minutes. Insoluble material was removed by centrifugation at 10,000 rpm for 10 minutes at 4°C. For immunoblotting, approximately 30 μg of protein from each sample was separated on a 16.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose. After blocking overnight in a solution of TBS containing 2% nonfat dry milk and 1% Tween 20, membranes were incubated with the primary antibody in blocking buffer for 2 hours at room temperature. The bcl-2 antibody (6C8),12 a hamster monoclonal specific for human bcl-2, and the bcl-X, rabbit antiserum14 were used at dilutions of 1:200 and 1:1,000 respectively (both antibodies were a gift of Dr Stanley Korsmeyer). After washing, the bcl-2 immunoblot was sequentially incubated with a biotinylated goat-antihamster IgG antiserum (Caltag, San Francisco, CA) at a dilution of 1:2,000 and horseradish peroxidase (HRP)-conjugated streptavidin (Zymed, San Francisco, CA) at 1:20,000 dilution for both antibodies and washed. The slides were sequentially incubated with a sheep anti-goat IgG antibody and a biotinylated donkey anti-sheep IgG antibody. For the detection of bcl-2 and bcl-X, the slides were developed with the 3-aminophenylcarbazole substrate (Boehringer Mannheim, Indianapolis, IN) at a concentration of 1:1000, followed by an enhancement step using a mixture of 0.01 M diaminobenzidine tetrahydrochloride, 0.001 M hydrogen peroxide diluted in 0.05 M Tris-Cl, pH 7.4, followed by a final rinse in distilled water. Cells were spun onto glass slides as described above and fixed in 1% paraformaldehyde in TBS for 30 minutes at room temperature. Endogenous peroxidase activity was blocked by incubation of samples in 0.1% H2O2 in TBS for 30 minutes at room temperature. DNA termini were labeled by incubation at 37°C for 1 hour in a solution containing 5 μmol/L digoxigenin-conjugated dUTP (Boehringer Mannheim, Indianapolis, IN) and 400 μU/mL terminal deoxynucleotidyl transferase in buffer supplied by the manufacturer (Promega, Madison, WI). After washing in TBS, the slides were sequentially incubated with a sheep anti-digoxigenin Fab (Boehringer Mannheim) at a 1:200 dilution and an HRP-conjugated goat-antisheep IgG antibody (Zymed, San Francisco, CA) at a 1:120 dilution. Slides were developed with the 3-aminophenylcarbazole substrate per manufacturer’s recommendations (Zymed, San Francisco, CA).

RESULTS

Overexpression of bcl-2 or bcl-X, in 32D cells suppresses apoptosis induced by cytokine withdrawal. 32D cells comprise an IL-3-dependent cell line that rapidly undergoes apoptosis upon cytokine withdrawal.14 Expression of both bcl-2 and bcl-X, in 32D cells appears to be responsive to IL-3; mRNA levels for both genes decrease rapidly upon IL-3 removal.23 Bcl-2 overexpression has been shown to delay apoptosis in a number of cytokine-dependent cell lines upon cytokine deprivation.24-26 These data suggest that bcl-2, or a related family member, may mediate the survival signal provided by cytokines. Therefore, we tested the ability of bcl-2 and bcl-X, to suppress apoptosis in cytokine-deprived 32D cells. cDNAs for human bcl-2 or murine bcl-X, were cloned into the expression vector pBSRαEN, and the resulting constructs transfected into 32D cells. Three clones each were isolated that contained the bcl-2, bcl-X, or vector alone constructs (designated 32D-bcl2, 32D-bclX, or 32D-neo, respectively). The responses of the three 32D-bcl2 or 32D-bclX clones in our assays were nearly identical, therefore only representative results are shown. Immunoblots of cell lysates demonstrated the appropriate expression of either bcl-2 or bcl-X, (Fig 1). These clones were examined for their survival in serum containing medium without added IL-3.
Fig 4. Morphology of 32D-bc12 cells after cytokine deprivation (original magnification × 600). May-Grünewald-Giemsa staining was performed on the following cells. (A) 32D-bc12 cells in IL-3-containing medium. (B and D) 32D-neo and 32D-bc12 cells, respectively, cultured for 7 days in medium containing 10 ng/mL of G-CSF. (C) 32D-bc12 cells cultured for 7 days in medium without G-CSF. Necrotic cells were removed by density centrifugation.

Approximately 50% of 32D-bc12 or 32D-bclX cells survived 3 days after cytokine deprivation compared with no survival of 32D-neo cells (Fig 2). These data indicate that constitutive expression of bcl-2 or bcl-XL is able to prolong survival in IL-3–deprived 32D cells; further, this survival advantage is similar to that achieved with G-CSF stimulation of control cells (Fig 2; neo + G-CSF). Interestingly, stimulation of 32D-bc12 or 32D-bclX cells with G-CSF had an apparent synergistic effect on cell survival (Fig 3). A greater than fivefold higher number of viable cells were present in G-CSF–stimulated 32D-bc12 cultures when compared with either 32D-bc12 cultures without added cytokine or to 32D-neo cells cultured in G-CSF.

The phenotype of 32D cells stimulated with G-CSF is distinct from 32D-bc12 or 32D-bclX cells cultured in the absence of added cytokine. The stochastic model of cytokine-induced hematopoietic differentiation predicts that the phenotype of 32D-bc12 or 32D-bclX cells surviving in culture after cytokine deprivation should be identical to the phenotype of cells surviving in G-CSF–containing cultures. In the presence of IL-3, all clones had an identical myeloblastic phenotype (Fig 4A and data not shown). The morphology of cells surviving after 7 days in the indicated culture conditions is shown in Fig 4. 32D cells stimulated with G-CSF acquire many features of more mature myeloid cells, including nuclear segmentation and the appearance of cytoplasmic azurophilic granules (Fig 4B). 32D-bc12 cells show a similar degree of nuclear segmentation, but the cells are consistently smaller with no apparent granule formation (Fig 4C). 32D-bc12 cells retain the ability to differentiate in response to G-CSF (Fig 4D). Similar results were obtained with 32D-bclX cells (data not shown).

G-CSF treatment of 32D cells is associated with the expression of a number of proteins found in the primary (azurophilic) and secondary granules of myeloid cells. The apparent lack of cytoplasmic granules in 32D-bc12 or 32D-bclX cells surviving cytokine deprivation suggested that expression of granule constituent proteins may be defective. Therefore, we examined cultured cells for their expression of myeloperoxidase, an enzyme found in the primary granules of myelomonocytic cells. Over 75% of 32D-neo and 32D-bc12 cells present after 7 days of G-CSF stimulation demonstrated myeloperoxidase activity (Fig 5B and D). In contrast, less than 5% of 32D cells grown in IL-3–containing medium or 32D-bc12 cells surviving in cultures containing no added cytokines were myeloperoxidase positive (Fig 5A and C). Similar results were obtained with 32D-bclX cells (data not shown).

Expression of cathepsin G mRNA is induced in 32D cells stimulated with G-CSF but not in 32D-bc12 or 32D-bclX cells surviving cytokine deprivation. Cathepsin G is a he-
matopoietic serine protease found in the primary granules of myelomonocytic cells. Its mRNA expression is restricted to cells at the promyelocyte stage of myelomonocytic maturation. Treatment of 32D-neo or 32D-bc12 cells with G-CSF for 3 days resulted in the accumulation of cathepsin G mRNA (Fig 6, lanes 2 and 5). No increase in the level of cathepsin G transcripts is seen in 32D-bc12 cells surviving cytokine deprivation (Fig 6, compare lanes 3 and 4). Equivalent amounts of β-actin mRNA were detected in each sample, indicating that the differences in cathepsin G mRNA expression observed in this assay are not caused by differences in RNA amount or integrity.

DISCUSSION

Hematopoietic cytokines are polypeptide soluble factors that control the growth and differentiation of hematopoietic cells. These cytokines are capable of stimulating the proliferation and enhancing the survival of the appropriate hematopoietic progenitor cell. However, the mechanisms by which cytokines promote hematopoietic differentiation remain controversial. Two general models for the role of cytokines in controlling hematopoietic differentiation have been proposed. In the stochastic model, hematopoietic growth factors provide proliferative and survival signals to the differentiating hematopoietic cell, but they do not provide specific lineage-commitment signals. In the instructive model, hematopoietic growth factors provide lineage commitment, along with survival and proliferative signals. In this study, we tested these two models with respect to G-CSF and granulocytic differentiation. We used 32D cells, a cell line capable of differentiating into granulocytes in response to G-CSF. The stochastic model predicts that 32D cells are already lineage committed and under permissive culture conditions will undergo granulocytic differentiation without G-CSF. Therefore, we generated 32D clones that constitutively expressed either bcl-2 or bcl-XL in an effort to circumvent the cell survival signals normally provided by G-CSF. The ability of these clones to undergo granulocytic differentiation in the absence of G-CSF was then determined.

Bcl-2 is the prototype of a family of proteins that functions to repress programmed cell death in a variety of cell lines. Bcl-2 can suppress apoptosis in a number of IL-3-dependent hematopoietic cell lines upon IL-3 withdrawal, including 32D cells. Our data confirm that both bcl-2 and bcl-XL are capable of suppressing apoptosis in cytokine deprived 32D cells. Prolonged survival, up to 10 days, in IL-3-deprived cultures was noted (data not shown) and was similar to that observed in G-CSF-stimulated cultures. Interestingly, stimulation of 32D-bc12 or 32D-bc1 XL cells with G-CSF had a synergistic effect on cell survival. This synergism
Apoptotic cells were detected infrequently; further, no correlation between nuclear segmentation and apoptosis was observed (data not shown). In the presence of G-CSF, 32D-bcl2 and 32D-bclX clones express myeloperoxidase and cathepsin G, proteins found in the primary granules of myelomonocytic cells. No evidence for an increased expression of these proteins was detected in the absence of G-CSF. These data support a hybrid model for the role of G-CSF in granulocytic differentiation; although some features of granulocytic differentiation, namely nuclear segmentation, do not require G-CSF and therefore appear to be preprogrammed in 32D cells, the complete maturation of 32D cells to granulocytes appears to be dependent on G-CSF.

Fairbairn et al. reported that FDCP-Mix cells constitutively expressing bcl-2 underwent multilineage hematopoietic differentiation in the absence of exogenous cytokine. Two criteria were used to assess for granulocytic differentiation, the expression of lysozyme M and the cellular morphology after May-Grünwald-Giemsa staining. Lysozyme M is expressed predominantly in mature myelomonocytic cells; however, in this study expression was induced in FDCP-Mix cells undergoing predominantly erythroid differentiation, suggesting that in this system lysozyme M expression may not be a reliable indicator of granulocytic differentiation. On the other hand, a distinguishing feature of mature neutrophils is the segmentation of their nuclei; therefore, the appearance of cells with segmented nuclei in their serum-free cultures is indicative of granulocytic differentiation and consistent with the hypothesis that the process of nuclear segmentation that occurs during granulopoiesis is independent of G-CSF.

In the present study, we show that several features associated with granulocytic differentiation in 32D cells are dependent on G-CSF, namely induction of myeloperoxidase activity and increased cathepsin G expression. These data suggest that G-CSF, through its receptor, is transmitting specific maturation signals. This hypothesis is consistent with recent reports describing truncation mutations of the G-CSFR that are unable to mediate differentiation responses.9,12,13 Work is in progress to define the structural motifs of the G-CSFR necessary for the differentiation response.

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JE Rodel and DC Link