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Notch Signaling Enhances Survival and Alters Differentiation of 32D Myeloblasts

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The Notch transmembrane receptors play important roles in precursor survival and cell fate specification during hematopoiesis. To investigate the function of Notch and the signaling events activated by Notch in myeloid development, we expressed truncated forms of Notch1 or Notch2 proteins that either can or cannot activate the core binding factor 1 (CBF1) in 32D (clone 3) myeloblasts. 32D cells proliferate as blasts in the presence of the cytokines, GM-CSF or IL-3, but they initiate differentiation and undergo granulopoiesis in the presence of granulocyte CSF (G-CSF). 32D cells expressing constitutively active forms of Notch1 or Notch2 proteins that signal through the CBF1 pathway maintained significantly higher numbers of viable cells and exhibited less cell death during G-CSF induction compared with controls. They also displayed enhanced entry into granulopoiesis, and inhibited postmitotic terminal differentiation. In contrast, Notch1 constructs that either lacked sequences necessary for CBF1 binding or that failed to localize to the nucleus had little effect. Elevated numbers of viable cells during G-CSF treatment were also observed in 32D cells overexpressing the basic helix-loop-helix protein (bHLH), HES1, consistent with activation of the CBF1 pathway. Taken together, our data suggest that Notch signaling enhances 32D cell survival, promotes entry into granulopoiesis, and inhibits postmitotic differentiation through a CBF1-dependent pathway. The Journal of Immunology, 2000, 165: 4428–4436.

The Notch cell surface receptors have been shown to play important roles in a broad spectrum of cell fate specifications during metazoan development (1–5). In many cases, Notch signaling regulates cell fate choices of bipotential precursors through lateral specification resulting from interactions of Notch with other intrinsic cellular factors and other signaling pathways. In general, Notch activity modulates the response of a precursor cell to multiple environmental signals. This response is frequently manifested as an inhibition of progenitor cell differentiation (6, 7). Recent studies also suggest that Notch signaling inhibits apoptosis (8–10) and promotes cell proliferation (11, 12). Overall, Notch function is highly dependent on the developmental context.

The Notch family of receptors (Notch1–4) consists of large transmembrane proteins. The extracellular domain of Notch is composed of 36 epidermal growth factor (EGF)3-like repeats and three Lin-12/Notch repeats; the intracellular domain consists of six ankyrin-like repeats with two flanking nuclear localization sequences, a core binding factor 1 (CBF1) interaction region (RAM domain), and a proline-glutamate-serine-threonine-rich (PEST) motif. In Drosophila and mammalian systems, constructs expressing an isolated intracellular domain of Notch result in a constitutively active, gain-of-function phenotype (13–15). Genetic and biochemical studies suggest that signaling by this activated form of Notch involves the transcriptional regulator, CBF1 (the mammalian homolog of Drosophila suppressor of hairless) (16–18). Further downstream, CBF1 up-regulates the expression of a basic helix-loop-helix (bHLH) transcription factor, HES1 (the mammalian homolog of the Drosophila hairy enhancer of split) (19, 20) to repress lineage-specific genes such as achaete-scute in neurons (21). Recently, this simple concept of Notch signal transduction has been challenged in an in vitro myeloblast system in which Notch signaling inhibits muscle cell differentiation through both CBF1-dependent and -independent pathways (22). Noziger et al. (23) suggested that the CBF1-independent pathway provides a general block in cell differentiation that is reinforced by cell type-specific, CBF1-dependent signals.

The hematopoietic system provides an excellent model for studying the function of Notch in cell fate specification, because hematopoiesis requires continuous progenitor cell proliferation, lineage commitment, differentiation, and maturation. Considerable evidence suggests that Notch plays critical roles in several steps of hematopoiesis. The best characterized examples are in lymphoid development. The TAN-1/Notch1 translocation (7, 9) (q34;q34.3) results in an active form of Notch1 mobilized to the TCR locus, and is responsible for the formation of a subset of acute T-lymphoblastic leukemias in humans (24). Transplantation of retrovirally transduced bone marrow cells expressing activated forms of Notch induces T cell leukemia and influences B- vs T-lineage determination in mice (25, 26). Studies of transgenic mice over-expressing an activated form of Notch1 in developing T cells suggest that Notch1 participates in the CD4 vs CD8 and the αβ vs γδ decisions (27, 28). A conditional knockout of Notch1 in newborn mice results in a severe deficiency in thymocyte development (29). Furthermore, activation of the Notch signaling pathway confers
resistance to TCR-mediated (9) and glucocorticoid-induced (8) apoptosis in CD4⁻/CD8⁻ thymocytes and up-regulates cellular markers correlating with maturation (8).

In myeloid development, Notch signaling has been reported to inhibit or delay differentiation of the 32D myeloblast cell lines (7, 30, 31) and HL-60 (32), and we and others have shown it to increase the formation of primitive precursor cell populations from humans and mice (33, 34). The conditional knockout of Notch1 did not yield obvious anomalies in myeloid development (29), perhaps because of the functional redundancy between Notch1 and Notch2, or because Notch2 may be the predominant form in myeloid progenitor cells, as is suggested by its prevalence in 32D myeloid progenitor cells (35). Furthermore, transplantation of bone marrow cells overexpressing active forms of Notch1 did not yield an apparent granulocytic phenotype, as assessed by FACS analysis of Mac1⁺/Gr1⁺ cells (26), probably because such cells represent a pool of myeloid cells without distinguishing stages of differentiation during myelopoiesis. Clearly, a more detailed analysis of Notch function in the progression of myeloid differentiation is needed.

To gain further insight into the function of Notch signaling during hematopoiesis in general, and specifically in myelopoiesis, we began to study the function of Notch in 32D (clone 3) myeloblast differentiation. In addition, we examined the Notch1/CBF1/HES1 signaling pathway in these cells. We chose to use the well-characterized cell line, 32D (36), because these cells closely resemble bipotent granulocyte-macrophage progenitors in bone marrow. Originally derived from normal murine bone marrow, 32D cells are diploid and not leukemic in syngeneic murine recipients. Similar to CFU-GM, these cells have both granulocytic and monocytic potential (37). The cytokines, GM-CSF and IL-3, support their proliferation, whereas granulocyte CSF (G-CSF) induces granulopoiesis and maturation, followed by cell cycle arrest. We generated 32D cell stable populations expressing truncated forms of Notch1 or Notch2 that can or cannot signal through CBF1 to examine the effect of Notch activation on 32D cell survival and differentiation. Our results suggest that Notch1 and Notch2 enhance 32D cell survival, promote initial granulopoiesis, and inhibit postmitotic differentiation through a CBF1-dependent pathway.

**Materials and Methods**

*Preparation of cellular RNA and RT-PCR*

Total cellular RNA was isolated from 32D cells (clone 3), a factor-dependent murine myeloid cell line (generously provided by Dr. Joel Greenberger, University of Pittsburgh, Pittsburgh, PA) using RNeasy reagents (Tel-Test, Friendswood, TX) according to the manufacturer’s protocol. PCR primers were designed to be specific to nonhomologous regions of Notch1 and Notch2 extracellular domains. The primer sequences for Notch1 and Notch2 were 5'-gccagtcattgagagacct and 3'-accattggtgcaagtagca; and the primers for Notch2 were 5'-accagccccctgcctgctac and 3'-ctctgccgcctctcag. RT-PCR was performed using Titan RT-PCR reagents (Roche, Indianapolis, IN). To eliminate genomic DNA contamination, total RNA was treated with DNase I (Life Technologies, Grand Island, NY). Reactions lacking reverse transcriptase were used as negative controls. Reverse transcription was conducted at 65°C for 30 min. PCRs were conducted in a thermal cycler (PTC100; MJ Research, San Francisco, CA) for 36 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

*Flow cytometry for detecting Notch1 and Notch2 expression*

32D cells were washed in PBS, fixed in 0.5% paraformaldehyde for 20 min at room temperature, then permeabilized in 70% EtOH for 10 min at 4°C. After two PBS washes, cells were resuspended in PBS, pH 7.2, containing 5 mM EDTA and 0.5% BSA (Sigma, St. Louis, MO) and allowed to rehydrate for 30 min at 37°C. Cells (10⁹) were then treated with 1 µg anti-CD32 mAb (PharMingen, San Diego, CA) for 10 min at room temperature, then incubated for 30 min with 0.4 µg of affinity-purified rabbit antiserum to either the intracellular domain of rat Notch1 (93-4) or Notch2 (93-7) or with an equivalent amount of normal rabbit IgG (Sigma). After PBS washes, cells were stained for 15 min with a 1:100 dilution of human-adsorbed FITC-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA). Cells were washed again and analyzed immediately on a FACScan flow cytometer, using Cell Quest software (Becton Dickinson, Mountain View, CA).

cDNA expression constructs

The truncated forms of Notch used in our work have been described previously (22, 23). Briefly, deletion mutants of Notch1 and Notch2 cDNA (see Fig. 2A) were cloned in a retroviral vector, SRαMSV/KNeo (generously provided by Dr. Owen Witte, University of California at Los Angeles, Los Angeles, CA), to encode the following amino acids: ZEDN1, 1–23 plus 1712–2531; OEDN1, 1–23 plus 24–60 and 1712–2531; CDN1, 1848–2531; and CDN2, 1786–2472. The truncated forms of Notch used in our work have been described previously (22, 23). Briefly, deletion mutants of Notch1 and Notch2 cDNA (see Fig. 2A) were cloned in a retroviral vector, SRαMSV/KNeo (generously provided by Dr. Owen Witte, University of California at Los Angeles, Los Angeles, CA), to encode the following amino acids: ZEDN1, 1–23 plus 1712–2531; OEDN1, 1–23 plus 24–60 and 1712–2531; CDN1, 1848–2531; and CDN2, 1786–2472. The truncated forms of Notch used in our work have been described previously (22, 23). Briefly, deletion mutants of Notch1 and Notch2 cDNA (see Fig. 2A) were cloned in a retroviral vector, SRαMSV/KNeo (generously provided by Dr. Owen Witte, University of California at Los Angeles, Los Angeles, CA), to encode the following amino acids: ZEDN1, 1–23 plus 1712–2531; OEDN1, 1–23 plus 24–60 and 1712–2531; CDN1, 1848–2531; and CDN2, 1786–2472. The truncated forms of Notch used in our work have been described previously (22, 23). Briefly, deletion mutants of Notch1 and Notch2 cDNA (see Fig. 2A) were cloned in a retroviral vector, SRαMSV/KNeo (generously provided by Dr. Owen Witte, University of California at Los Angeles, Los Angeles, CA), to encode the following amino acids: ZEDN1, 1–23 plus 1712–2531; OEDN1, 1–23 plus 24–60 and 1712–2531; CDN1, 1848–2531; and CDN2, 1786–2472. The truncated forms of Notch used in our work have been described previously (22, 23). Briefly, deletion mutants of Notch1 and Notch2 cDNA (see Fig. 2A) were cloned in a retroviral vector, SRαMSV/KNeo (generously provided by Dr. Owen Witte, University of California at Los Angeles, Los Angeles, CA), to encode the following amino acids: ZEDN1, 1–23 plus 1712–2531; OEDN1, 1–23 plus 24–60 and 1712–2531; CDN1, 1848–2531; and CDN2, 1786–2472. The truncated forms of Notch used in our work have been described previously (22, 23). Briefly, deletion mutants of Notch1 and Notch2 cDNA (see Fig. 2A) were cloned in a retroviral vector, SRαMSV/KNeo (generously provided by Dr. Owen Witte, University of California at Los Angeles, Los Angeles, CA), to encode the following amino acids: ZEDN1, 1–23 plus 1712–2531; OEDN1, 1–23 plus 24–60 and 1712–2531; CDN1, 1848–2531; and CDN2, 1786–2472. The truncated forms of Notch used in our work have been described previously (22, 23). Briefly, deletion mutants of Notch1 and Notch2 cDNA (see Fig. 2A) were cloned in a retroviral vector, SRαMSV/KNeo (generously provided by Dr. Owen Witte, University of California at Los Angeles, Los Angeles, CA), to encode the following amino acids: ZEDN1, 1–23 plus 1712–2531; OEDN1, 1–23 plus 24–60 and 1712–2531; CDN1, 1848–2531; and CDN2, 1786–2472.
Twenty-four hours after transduction or electroporation, cell populations CA). This strategy was used to maximize the level of HES1 expression.

Retroviral transduction and transient transfections
Infectious retroviral supernatants were produced by simultaneously transfecting (CalPhos Maximizer Transfection Kit; Clontech Laboratories) 293T cells with two plasmids, one encoding a deletion form of Notch in the SRα retroviral vector, and the other encoding the ecotropic helper virus, pCDNA3. 32D cells (10⁶/ml) were subsequently incubated in 1 ml of retroviral supernatant in 2 ml of growth medium (see Cell culture), and 2 μg/ml polybrene (hexadimethrine bromide; Sigma) at 37°C for 24 h. Cell populations over-expressing HES1 were generated by electroporating 30 μg of the pBos-EF12-HES1 plasmid (provided by Dr. Riyoichiro Kageyama of Kyoto University, Kyoto, Japan) with 3 μg of the pCDNA3 plasmid (Invitrogen, San Diego, CA) at 250 V, 960 μF, using a Gene Pulser (Bio-Rad, Richmond, CA). This strategy was used to maximize the level of HES1 expression. Twenty-four hours after transduction or electroporation, cell populations stably expressing the desired proteins were selected and maintained in 1.2 mg/ml of genetin (G418; Life Technologies).

For subcellular localization studies, 3 μg of pEGFP-ZEDN1, pEGFP-OEDN1, pEGFP-CDN1, pEGFP-CDN2, or pEGFP vector control was transfected into NIH 3T3 cells (no. 1658-CRL; American Type Culture Collection, Manassas, VA) using CalPhos Maximizer Transfection reagents (Clontech). Photographs were taken at ×400 magnification, using an Olympus (New Hyde Park, NY) fluorescence microscope (model IX50) at 24 h posttransfection.

Cell culture
32D cells were maintained in IMDM supplemented with 10% FBS (Omega Scientific, Tarzana, CA), 0.4 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 200 μM uridine GM-CSF (a gift from Amgen, Thousand Oaks, CA). When growing in GM-CSF culture, cells were split 1:100 every 3 days. To differentiate cells, 32D stable cell populations (1.6 × 10⁶ cells/ml) were washed three times in medium to remove residual GM-CSF, then cultured in medium supplemented with 50 ng/ml of human G-CSF (provided by Amgen). After a time course of 4 days, the number of viable cells, the percentage of dead cells, and the percentage of differentiation were evaluated. The numbers of viable cells were evaluated by trypsin blue exclusion using a hemacytometer. Equal culture volumes were centrifuged onto individual slides using a Cytospin 3 (Shandon, Pittsburgh, PA). Cytospin slides were stained with a Diff Quick Set (Dade Diagnostics, Aguada, PR) and evaluated in a blinded fashion for granulocytic differentiation. The criteria for differentiation included nuclear segmentation, cytoplasm-nucleus ratio, and granularity. The differential counts were performed on 300 cells per slide for blasts, promyelocytes, myelocytes, metamyelocytes, mature granulocytes, and dead cells.

Western blot analysis
Cells (3 × 10⁵ per lane) were lysed and electrophoresed on a 7.5% or 10% SDS-PAGE mini-gel. Separated proteins were transferred to a Hybond nitrocellulose membrane (Amersham, Arlington Heights, IL) and blocked with 5% nonfat dry milk in TBS containing 1% Tween 20 for 2 h at room temperature. Membranes were then incubated with one of the three polyclonal rabbit antibodies for 2 h at room temperature. The 93–4 antisense (22) against the intracellular domain of rat Notch1 was used at a dilution of 1:4000, the 93–7 antisense (38) against the intracellular domain of rat Notch2 was affinity-purified and used at ~32 μg/ml, and the IgG fraction of the HES1 antisemur (a gift from Dr. Tetsuo Sudo, Toray Industries, Osaka, Japan) was used at 3.6 μg/ml. Immunoreactivity was detected using a biotinylated donkey anti-rabbit IgG Ab (Amersham), HRP-conjugated streptavidin (Amersham), and enhanced chemiluminescence Western blot reagents (Amersham).

TUNEL assay
Twenty-four hours after G-CSF induction (see Cell culture), 32D cell populations were washed twice with PBS containing 1% BSA and fixed for 1 h with 4% paraformaldehyde in PBS, pH 7.4. After washing with PBS twice, cells were permeabilized for 2 min with 0.1% Triton X-100 in 0.1% sodium citrate. Cells were then washed in PBS and resuspended in TUNEL reaction mixture for 1 h at 37°C according to the manufacturer’s protocol. After two washes in PBS, cells were analyzed by flow cytometry.

CBF1 trans-activation assay
32D cells (10⁵) were electroporated with 30 μg of the indicated Notch deletion construct (see Fig. 2A), along with 10 μg of 4×CBF1Luc (16) and 1 μg of the β-galactosidase plasmid pCH110 (Pharmacia, Piscataway, NJ). Twenty-four hours postelectroporation, cells were evaluated for both luciferase and β-galactosidase activity according to the manufacturer’s instructions (Promega, Madison, WI). β-galactosidase expression was used to control for differences in transfection efficiencies. CBF1 activity is reported as fold increase in normalized luciferase values for each Notch construct relative to vector control values.

Results
32D cells express endogenous Notch1 and Notch2
To confirm a previous report of endogenous Notch expression in 32D cells (7), we used RT-PCR and flow cytometric analysis to detect both Notch1 and Notch2. Because Notch1 and Notch2 are highly homologous, PCR primers were designed against unique regions of either Notch1 or Notch2, and primer specificity was verified using Notch1 or Notch2 plasmid cDNAs. Fig. 1A shows that the Notch1 primers only amplified Notch1 cDNA (lane 2) and

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** Schematic representation of full-length and truncated Notch1 and Notch2 proteins, and Western blot analysis of these proteins expressed in stable 32D populations. A, Full-length Notch1 or Notch2 extracellular domain consists of 36 tandem EGF-like repeats required for ligand binding, and three Lin-Notch repeats characteristic of the *Caenorhabditis elegans* protein, LIN-12, and all Notch proteins. TM, Transmembrane domain. The cytoplasmic domain of Notch contains six repeats with homology to the ankyrin (ANK) protein. The region between the transmembrane domain and the ankyrin repeats has been designated the RAM domain. The cytoplasmic domain of Notch may be important for protein stability (see Materials and Methods for the specific amino acids encoded by the ZEDN1, OEDN1, CDN1, and CDN2 constructs). B, Western blot analysis of ZEDN1, OEDN1, CDN1, and CDN2 protein expression in 32D cells. Total cellular protein extracts were prepared from stable 32D populations. The Western blots were probed with either Notch1 (lanes 1–4) or Notch2 antisemur (lanes 5–6). Arrows indicate the expression of CDN1 (lane 2), OEDN1 (lane 3), ZEDN1 (lane 4), and CDN2 (lane 6). Lanes 1 and 5, SRα vector controls.
Subcellular localization of ZEDN1-, OEDN1-, CDN1-,
and CDN2-EGFP fusion proteins. a (bright field) and b (corresponding
dark field), Expression of the EGFP vector. c and d, Expression of the
ZEDN1-EGFP fusion protein. e and f, Expression of the OEDN1-EGFP fusion protein.
g and h, Expression of the CDN1-EGFP fusion protein. i and j, Expression of the CDN2-EGFP fusion protein. Cells were visualized under
fluorescent microscopy at 24 h posttransfection.

FIGURE 3. Subcellular localization of the ZEDN1-, OEDN1-, CDN1,
and CDN2-EGFP fusion proteins. a (bright field) and b (corresponding
dark field), Expression of the EGFP vector. c and d, Expression of the
ZEDN1-EGFP fusion protein. e and f, Expression of the OEDN1-EGFP fusion protein.
g and h, Expression of the CDN1-EGFP fusion protein. i and j, Expression of the CDN2-EGFP fusion protein. Cells were visualized under
fluorescent microscopy at 24 h posttransfection.

not Notch2 cDNA (lane 3). Conversely, the Notch2 primers were
specific to Notch2 cDNA (lane 5) and did not amplify Notch1
cDNA (lane 4). In RT-PCR analysis, endogenous RNA species for
Notch1 and Notch2 were detected in lanes 7 and 9, respectively.
No bands were detected in the absence of RT enzyme (lanes 6 and
8), indicating that there was no genomic DNA contamination. Fig.
1B shows flow cytometric analysis of 32D cells stained with an-
tiserum against intracellular Notch1 or Notch2, indicating the pres-
ence of endogenous Notch1 and Notch2 protein isoforms.

Stable 32D cell populations expressing truncated forms of
Notch1 or Notch2 proteins

To identify structural motifs important for Notch function in 32D
cells, we used deletion constructs of Notch1 (OEDN1, ZEDN1,
CDN1) and Notch2 (CDN2) (see Fig. 2A). OEDN1 and ZEDN1
encode the signal peptide, the transmembrane domain, and the en-
tire intracellular domain of Notch1. In addition, OEDN1 encodes
one and a half extracellular EGF repeats. Both ZEDN1 and
OEDN1 contain the entire 117 amino acids of the RAM domain,
which is located between the transmembrane domain and the
ankyrin repeats, and is required for CBF1 binding. CDN1, which
initiates 17 amino acids upstream of the ankyrin repeats, lacks
most of the RAM sequences necessary for productive CBF1 in-
teractions. CDN2, which initiates 36 amino acids upstream of the
ankyrin repeats of Notch2, encodes the RAM 1b domain (a weak
CBF1 interaction domain), but not the stronger affinity 1a domain
(16, 38).

Protein expression was confirmed by Western analysis of 32D-
SRs, 32D-ZEDN1, 32D-OEDN1, 32D-CDN1, and 32D-CDN2
stable populations, using antisera raised against the cytoplasmic
sequences of Notch1 or Notch2. The CDN1 and CDN2 proteins
(lanes 2 and 6), with approximate molecular masses of 75 kDa, as
well as OEDN1 (lane 3) and ZEDN1 (lane 4) proteins, with ap-
proximate molecular masses of 97 and 93 kDa, respectively, are
indicated by arrows in Fig. 2B. The level of ZEDN1 expression
was consistently lower than that of OEDN1, CDN1, and CDN2.

Subcellular localization of ZEDN1, OEDN1, CDN1, and CDN2

The only difference between constructs ZEDN1 and OEDN1 is the
presence of a sequence encoding one and a half extracellular EGF
repeats in OEDN1. It is known that full-length Notch must be
proteolytically processed at two extracellular sites and one trans-
membrane/intracellular site before full activation and nuclear lo-
calization (39–42). We hypothesized that the one and a half extra
EGF-like repeats in the extracellular domain of OEDN1 might
affect OEDN1 processing and subcellular localization. For this rea-
son, we examined the subcellular localization of all the Notch con-
structs to be characterized in the biological assay.

To elucidate the subcellular localization of ZEDN1, OEDN1,
CDN1, and CDN2, we created the EGFP-fusion constructs, and
transfected NIH 3T3 cells either with the EGFP vector or with
sequences encoding fusion constructs. When the pEGFP
vector was transfected, the green fluorescence was distributed throughout
the entire cell (Fig. 3b). The EGFP-ZEDN1 protein was predom-
antly localized in the cell nucleus (Fig. 3d), with aggregates pos-
sibly located in the endoplasmic reticulum and the Golgi complex.
In contrast, Fig. 3f shows that the EGFP-OEDN1 protein did not local-
ize to the cell nucleus, suggesting that the processing of
OEDN1 was abnormal, preventing its nuclear import. Fig. 3, h and
j, shows that CDN1-EGFP and CDN2-EGFP were found in both
the cytoplasm and the nucleus.

Intracellular forms of Notch1 and Notch2 that contain all or
part of the RAM region enhance survival of 32D cells in the
presence of G-CSF

To investigate the effects of overexpression by various forms of
Notch in granulopoiesis, 32D-SR, 32D-ZEDN1, 32D-OEDN1, 32D-CDN1,
and 32D-CDN2 cell populations were cultured in
growth or differentiation conditions, and the numbers of viable and
dead cells, as well as the apoptosis profile, were evaluated. As
shown in Fig. 4A, when the cells were cultured in growth medium
supplemented with the cytokine GM-CSF, all five cell populations
proliferated at a similar rate, indicating that overexpression of the
various Notch1 and Notch2 proteins did not alter their growth
properties in GM-CSF.

To study the function of Notch signaling in 32D differentia-
tion, 32D stable populations were washed to remove GM-CSF, then
cultured in medium supplemented with the cytokine G-CSF, which
FIGURE 4. The effects of Notch overexpression on the growth and differentiation properties of 32D cells. A, 32D-SRα, 32D-ZEDN1, 32D-OEDN1, 32D-CDN1, and 32D-CDN2 stable cell populations were grown in GM-CSF culture. Cell density and viability were enumerated daily with a hemacytometer and trypan blue dye exclusion. B, 32D populations expressing the ZEDN1 and CDN2 proteins, which contain the entire or partial RAM domain, were able to maintain higher numbers of viable cells in G-CSF when compared with the SRα control. 32D-OEDN1 cells show a slight increase. 32D-CDN1 cells do not differ significantly from the vector control. A representative experiment performed in triplicate is shown. Error bars represent SEM. C, Cytospin preparations of 32D cells. a, 32D-SRα in GM-CSF culture; b–f, 32D cells at day 2 in G-CSF culture. An equal volume of culture was taken from 32D-SRα (b), ZEDN1 (c), OEDN1 (d), CDN1 (e), and CDN2 (f) for cytospins. ZEDN1 and CDN2 populations contained greater numbers of viable cells than the SRα, OEDN1, and CDN1 groups. D, 32D-ZEDN1 and 32D-CDN2 stable cell populations had lower percentages of dead cells in the presence of G-CSF. Dead and viable cells were enumerated daily. A representative experiment performed in triplicate is shown. Error bars represent SEM. E, 32D-ZEDN1 and 32D-CDN2 had lower percentages of apoptosis at day 1 in G-CSF culture. Apoptosis was assessed by TUNEL reagents. A representative experiment is shown.
provides a physiologically relevant differentiation signal for granulopoiesis. All cell populations used for the experiments were either freshly transduced or freshly thawed from frozen stocks. Over a time course of 4 days, the numbers of viable and dead cells, as well as the progression of differentiation, were analyzed. Fig. 4B demonstrates that the cells overexpressing ZEDN1 and CDN2 maintained higher numbers of viable cells compared with the 32D-SRα control on days 1–4 (mean ± SEM of triplicates). ZEDN1 exhibited the strongest phenotype, although it was expressed at a lower level than the other Notch constructs, suggesting that the observed phenotype did not result directly from variations in protein expression levels. Only a modest increase in cell numbers was observed in the 32D-OEDN1 population. 32D-CDN1 did not show a significant difference in total viable cell numbers compared with the SRα control. Fig. 4C shows the cytospin preparations of G-CSF cultures on day 2. The cytospins were conducted with an equal volume of culture from each group rather than with an equivalent cell number. Therefore, the cell number differences seen on the slides reflect the cell densities of the cultures. Again we observed that the numbers of viable cells in G-CSF culture were highest among the ZEDN1 (Fig. 4C, c) and CDN2 (Fig. 4C, f) populations.

To determine whether the observed increases of viable cells in 32D-ZEDN1 and 32D-CDN2 were due to enhanced survival of these cells in the presence of G-CSF, we calculated the number of dead cells as a percentage of total cells in all five groups. Fig. 4D shows that 32D-ZEDN1 and 32D-CDN2 stable populations had a significantly lower percentage of cell death compared with the 32D-SRα control on days 1–3. The 32D-OEDN1 population had a slight decrease in cell death on days 1 and 2, whereas the 32D-CDN1 population did not differ from the vector control.

Fig. 4E shows a TUNEL analysis assessing apoptosis in the stable cell populations at day 1 in G-CSF culture. The 32D-ZEDN1 population had less than half the levels of apoptosis (24%) compared with the vector control (53%). 32D-CDN2 cells also displayed lower levels of apoptosis (37%), whereas 32D-OEDN1 and 32D-CDN1 cells exhibited minimal alterations in apoptosis. These trends were seen in two separate experiments and were confirmed by Annexin V staining (data not shown).

From these results, we concluded that active forms of Notch containing the entire RAM domain (ZEDN1) or a partial RAM domain (CDN2) enhanced cell survival and inhibited apoptosis of 32D myeloblasts in G-CSF culture.

Dual effects of Notch signaling on 32D differentiation

The course of granulopoiesis in 32D cells progresses sequentially from undifferentiated blasts to promyelocytes, myelocytes, then metamyelocytes, and finally mature granulocytes, followed by cell cycle arrest. To examine the role of enforced Notch expression in the progression of 32D cell differentiation, we analyzed the percentage of differentiation for each stage of granulopoiesis. The cell populations (shown in Fig. 4C) were grouped into three categories: undifferentiated blasts, intermediate cells (including promyelocytes, myelocytes, and metamyelocytes), and mature granulocytes. The percentage of undifferentiated blasts remaining during G-CSF induction correlates inversely with the ability of the population to commit to granulopoiesis and differentiation. The percentage of mature granulocytes reflects the levels of postmitotic differentiation, the final step in differentiation of 32D cells.

Fig. 5A shows the percentage of undifferentiated blasts in G-CSF cultures. 32D-ZEDN1 and 32D-CDN2 cells displayed a lower percentage of blasts than the SRα control, whereas 32D-OEDN1 and 32D-CDN1 cells were not different from the control. We then examined the percentage of mature granulocytes in the culture. Fig. 5C demonstrates that 32D-ZEDN1 cells had a lower percentage of mature granulocytes than the SRα control. 32D-CDN2 cells had a similar though less profound phenotype, as compared with 32D-ZEDN1 cells. When compared with controls, more cells in the 32D-ZEDN1 and 32D-CDN2 populations progressed to promyelocytes, and a lower percentage of cells underwent terminal differentiation to mature granulocytes. Consequently, most of the cells in these populations accumulated in the early stages of differentiation as an expanded pool of promyelocytes, myelocytes, and metamyelocytes (Fig. 5B). 32D-OEDN1 and 32D-CDN1 cells had percentages of intermediate cells similar to the vector control.

Taken together, our data indicate that active forms of Notch containing the entire RAM domain (ZEDN1) or a partial RAM domain (CDN2) promoted entry to granulopoiesis and inhibited postmitotic differentiation of 32D cells in the presence of G-CSF.

FIGURE 5. Notch signaling exerted dual effects on 32D differentiation in the presence of G-CSF. A. 32D-ZEDN1 and 32D-CDN2 showed decreased percentages of undifferentiated blast cells at day 1 in G-CSF culture. A representative experiment in triplicate is shown. Error bars represent SEM. B. 32D-ZEDN1 and 32D-CDN2 exhibit increased percentages of intermediate cells including promyelocytes, myelocytes, and metamyelocytes at day 2 in G-CSF culture. A representative experiment in triplicate is shown. Error bars represent SEM. C. 32D-ZEDN1 and 32D-CDN2 exhibit decreased percentages of mature granulocytes at day 2 in G-CSF culture. A representative experiment in triplicate is shown. Error bars represent SEM. D. Schematic representation of the function of Notch on 32D differentiation. Cells were drawn to show the characteristic nuclear morphology during granulopoiesis. Corresponding stages of differentiation were marked below each cell, and an upward arrow indicates an increase in percentage, whereas a downward arrow indicates a decrease in percentage.
Notch deletion forms that contain the RAM domain or partial RAM domain can trans-activate endogenous CBF1

It has been reported that the RAM domain and the ankyrin repeats are required for CBF1 binding (43). Therefore, Notch constructs that lack almost the entire RAM domain, such as CDN1, should not activate CBF1. The interaction of endogenous CBF1 with Notch RAM domain sequences has not been tested in 32D cells. In addition, whether a construct such as OEDN1, which contains the entire RAM domain but is primarily localized outside the nucleus, can activate CBF1, has not been tested. To investigate the relationship of the Notch-induced survival and differentiation phenotype with CBF1 activity in 32D cells, we analyzed CBF1 trans-activation by ZEDN1, OEDN1, CDN1, and CDN2 in a CBF1-luciferase reporter system. When overexpressed in host cells, active forms of Notch that interact with endogenous CBF1 transform CBF1 from a transcriptional repressor to a transcriptional activator and drive the expression of the luciferase reporter gene.

Fig. 6 demonstrates that ZEDN1 strongly trans-activates CBF1 (~20-fold) in 32D cells, and CDN2 trans-activates CBF1 to a modest extent (~5-fold). Neither OEDN1 nor CDN1 show significant CBF1 trans-activation. The CBF1 trans-activation results described here correlate well with the cell survival and differentiation results described in the previous section, suggesting that Notch acts via a CBF1-dependent pathway in these cells.

32D cells overexpressing HES1 display a phenotype similar to ZEDN1 and CDN2

Genetic and biochemical studies suggest that Notch activation of CBF1 leads to up-regulation of a bHLH transcription factor, HES1 (19, 20). 32D cells express low levels of HES1, as seen by Northern analysis (data not shown). We postulated that if, in 32D cells, Notch signals via trans-activation of CBF1, which in turn up-regulates HES1, HES1-overexpressing cells should yield a phenotype similar to Notch-overexpressing cells. Therefore, we created 32D-HES1 stable cell populations and examined the differentiation properties of these cells. Fig. 7A shows that HES1 expression in 32D-HES1 cells was readily detectable, and Fig. 7B indicates the number of viable cells in 32D-HES1 and 32D-pBos cells on day 2 of G-CSF culture. The 32D-HES1 cells maintained higher numbers of viable cells in the presence of G-CSF than the vector control, similar to what was observed in 32D-ZEDN1 and 32D-CDN2 cells, suggesting that HES1 and Notch may function in the same pathway, and that HES1 may be a downstream effector of Notch in 32D cells. The 32D-HES1 phenotype was more subtle than that of 32D-ZEDN1 and 32D-CDN2, probably because HES1 is not the only effector of Notch. Therefore, we are probably only seeing a partial response of Notch activation when we overexpress HES1. For a subtle phenotype, such as that observed in 32D-HES1 cells, it was difficult to observe significant differences in differential counts (data not shown).

Discussion

In this study, we have shown that overexpression of active forms of Notch1 and Notch2 maintained higher numbers of viable cells, inhibited cell death, promoted initial granulopoiesis, and inhibited postmitotic differentiation of 32D myeloblasts in the presence of the cytokine G-CSF. The Notch RAM domain, which contains the CBF1 binding domain, was required for this function, suggesting that Notch signals through a CBF1-dependent pathway in these cells. Nuclear localization of Notch1 was also necessary for this function. Overexpression of HES1, a downstream target of CBF1, yielded a similar though less dramatic phenotype, further supporting our hypothesis that Notch signals via a CBF1-dependent pathway in 32D cells.

The dramatically higher numbers of viable cells and fewer dead cells in the 32D-ZEDN1 and 32D-CDN2 populations during G-CSF culture indicated that Notch signaling enhanced cell survival. The biphasic growth properties of 32D cells displayed in Fig. 4B reflected a window of adjustment for the 32D cells at days 1 and 2, before the cells began to proliferate transiently at days 3 and 4. The dramatic differences in viable cell numbers were largely due to differences in viable cell numbers at days 1 and 2, when the cells were in the nonproliferative period. 32D-ZEDN1 shortened the refractory period from 2 days to 1 day and, therefore, intensified
the differences in viable cell numbers. Further supporting the conclusion that Notch signaling enhanced 32D cell survival was that TUNEL analysis demonstrated that Notch signaling inhibited apoptosis during the initial 24 h of G-CSF treatment.

We observed a dual effect of Notch signaling on 32D differentiation (Fig. 5). In the presence of G-CSF, Notch signaling promoted progression from myeloblasts to promyelocytes, but inhibited postmitotic differentiation from metamyelocytes to mature granulocytes. Our data suggest that Notch signaling might be potentiating the G-CSF cytokine signal to initiate granulopoiesis. This action of Notch is consistent with other systems in which Notch signaling modulates the response of precursor cells to environmental signals (1–3). Because 32D cells resemble bipotent CFU-GM, which becomes committed to granulopoiesis when stimulated with G-CSF, it is possible that the initial lineage specification requires Notch to progress from myeloid blasts to promyelocytes.

Notch signaling inhibited postmitotic differentiation from metamyelocytes to mature granulocytes, as shown in Fig. 5C. This block to differentiation may contribute to fewer dead cells and more viable cells. The block to differentiation is consistent with Notch-mediated inhibition of granulocytic differentiation observed by Milner et al. (7) and with the oncogenic property of TAN-1 (translocation-associated Notch homolog) in T lymphoblastic leukemia (24, 25). However, we did not observe the expansion of undifferentiated 32D cells (namely, myeloblasts) observed by Milner et al. (7). In addition, these authors suggested that the active form of Notch1 inhibited 32D differentiation by G-CSF but not GM-CSF, whereas an active form of Notch2 inhibited differentiation induced by GM-CSF but not G-CSF (31).

In contrast, we did not observe an expansion of undifferentiated cells, but rather, the opposite; we observed that both Notch1 and Notch2 overexpression caused more cells to progress from myeloblasts to promyelocytes, resulting in a decreased percentage of undifferentiated cells (Fig. 5A). These conflicting observations could be due to differences in the cell populations under study. The 32D cells used in our investigation were regularly maintained in growth medium supplemented with GM-CSF, whereas the cells used by Milner et al. were regularly maintained in growth medium supplemented with WEHI-conditioned medium as a source of IL-3. In addition, there is inherent variability in the populations of 32D cells used in the two studies. Furthermore, Milner et al. used clones for their study, whereas we used freshly transduced cell populations. We also selected clones overexpressing an active form of Notch, and conducted G-CSF differentiation and cell survival experiments with similar results (data not shown).

With regard to structure-function analysis of Notch signaling, the current model suggests that there are three functional domains for the Notch intracellular region: the RAM domain and the ankyrin repeats necessary for CBF-1 binding and corepressor displacement, and the C-terminal trans-activation domain (43). Our CBF1 trans-activation data are consistent with this model. The CBF1 trans-activation results also correlate well with G-CSF survival and differentiation. 32D-ZEDN1, which contains all three functional domains, exhibited the strongest phenotype of enhanced survival and differentiation, and could strongly trans-activate CBF1. CDN2, which contains a partial RAM domain and showed an intermediate phenotype, could weakly trans-activate CBF1. OEDN1, due to its impaired nuclear localization, did not strongly activate CBF1, nor did it strongly affect 32D survival and differentiation. CDN1 does not contain the RAM domain and did not activate CBF1, nor did it yield phenotypes in G-CSF culture. The correlation of CBF1 trans-activation with enhanced survival and altered differentiation in 32D cells suggests that Notch functions through a CBF1-dependent pathway in these cells.

It is known that proteolytic release of the Notch intracellular domain is required for its function. One of the cleavages that results in the release of the Notch1 intracellular domain occurs within the transmembrane domain or immediately adjacent to it. There are recent reports demonstrating that presenilin is physically associated with Notch1 and may be involved in proteolytic cleavage (44–47). Our observation that the truncated Notch1 protein, OEDN1, was not localized to the nucleus suggests that OEDN1 was not processed correctly. The extra EGFR repeats may affect the conformation of OEDN1, such that proteolytic enzymes such as presenilin cannot access the cleavage site.

Tomita et al. did not conclude that the HES1 knockout mice showed a difference in Mac1+/Gr-1+ cells in fetal liver (11.1 vs 8.7%, and 12.5 vs 8.8%, respectively) (48). As is the case for Notch1 knockout mice, overlapping and redundant signaling pathways may preclude the generation of a robust myeloid phenotype in HES1−/− mice. In our experiments, overexpression of HES1 in 32D cells caused a phenotype similar to that caused by activated forms of Notch is consistent with the hypothesis that HES1 is a downstream target of Notch in these cells. Because HES1 is a bHLH protein, which usually interacts with other factors to influence transcription of downstream lineage-specific genes, we speculate that in 32D cells, HES1 may affect the activity of other transcriptional regulators to control the expression of myeloid-specific genes. Now that components of the Notch signaling pathway have begun to be characterized in hematopoietic cells, the next challenge is to identify downstream myeloid-specific genes regulated by the Notch/CBF1/HES1-dependent pathway.

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References

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