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Notch-1 Inhibits Apoptosis in Murine Erythroleukemia Cells and Is Necessary for Differentiation Induced by Hybrid Polar Compounds

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Abstract Strikingly increased expression of notch-1 has been demonstrated in several human malignancies and pre-neoplastic lesions. However, the functional consequences of notch-1 overexpression in transformed cells remain unclear. We investigated whether endogenously expressed notch-1 controls cell fate determination in mouse erythroleukemia (MEL) cells during pharmacologically induced differentiation. We found that notch-1 expression is modulated during MEL cell differentiation. Premature downregulation of notch-1 during differentiation, by antisense S-oligonucleotides or by enforced expression of antisense notch-1 mRNA, causes MEL cells to abort the differentiation program and undergo apoptosis. Downregulation of notch-1 expression in the absence of differentiation inducer increases the likelihood of spontaneous apoptosis. We conclude that in MEL cells, endogenous notch-1 expression controls the apoptotic threshold during differentiation and growth. In these cells, notch-1 allows differentiation by preventing apoptosis of pre-committed cells. This novel function of notch-1 may play a role in regulating apoptosis susceptibility in notch-1 expressing tumor cells. J. Cell. Biochem. 73:164–175, 1999.

Key words: differentiation-inducing drugs; apoptosis; cancer; notch-1

Genes of the notch family encode transmembrane receptors that are involved in cell fate decisions during development and postnatal life [Artavanis-Tsakonas et al., 1995; Blaumueller et al., 1997; Weinmaster, 1997; Greenwald, 1998]. Multiple, evolutionarily related notch genes have been described in mammals. Mature notch receptors are heterodimers derived from the cleavage of notch pre-proteins into an extracellular subunit (N^{EC}) containing multiple EGF-like repeats and a transmembrane subunit including the intracellular region (N^{TM}) [Blaumueller et al., 1997]. Notch activation results from the binding of ligands expressed by neighboring cells. Signaling from activated notch involves several pathways and accessory molecules, and remains incompletely understood [Greenwald, 1998; Weinmaster, 1997; Artavanis-Tsakonas et al., 1995; Jarriault et al., 1995; Shawber et al., 1996; Kopan et al., 1996]. Ligand-induced cleavage of the intracellular portion followed by nuclear access and interaction with transcription factors of the CSL family [CBF-1, Su(H), LAG-1] has been demonstrated in Drosophila [Struhl et al., 1998] and mammalian cells [Schroeter et al., 1998]. Expression of constitutively activated forms of notch receptors, lacking the all or most of the N^{EC} subunit, inhibits terminal differentiation in vitro in murine models of myogenesis and granulocytopoiesis [Kopan et al., 1994; Milner et al., 1996]. In chicken retina explants, a constitutively activated notch-1 inhibits differentiation of retinal progenitors to ganglion cells, while notch-1 antisense oligonucleotides increase differentiation towards a neuronal phenotype [Austin et al., 1995]. Overall, these studies suggest that in many systems notch activation inhibits or delays differentiation. In some experimental models, such as CD4/CD8

Abbreviations used: CSL, CBF-1, Suppressor of Hairless, Lag-1 family; HMBA, hexamethylene-bisacetamide; MEL, murine erythroleukemia; N^{TM}; notch-1 transmembrane subunit; N^{EC}, notch-1 extracellular subunit; S-oligo, phosphorothioate oligonucleotide; n1-AS, notch-1 antisense.

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and α/β vs. γ/δ lineage decisions in thymocytes [Robey et al., 1996; Washburn et al., 1997] and in vitro adipocyte differentiation of 3T3-L1 cells [Garces et al., 1997], expression of notch-1 appears to be necessary for proper interpretation of differentiation stimuli.

Several lines of evidence suggest that alterations of notch-1 signaling or expression contribute to oncogenesis. Deletions of the extracellular portion of human notch-1 are associated with about 10% of the cases of T cell acute lymphoblastic leukemia [Ellisen et al., 1991]. Truncated forms of notch-1 cause T cell lymphomas when introduced into mouse bone marrow stem cells [Pear et al., 1996] and can transform rat kidney cells in cooperation with the adenoviral oncogene E1A [Capobianco et al., 1997]. Additionally, strikingly increased expression of notch-1 has been documented in a number of human tumors including cervical, colon and lung carcinomas, and pre-neoplastic lesions of the uterine cervix [Zagouras et al., 1995; Daniel et al., 1997]. The possible function(s) of overexpressed notch-1 in transformed cells is unknown, nor is it known whether notch-1 can control cell fate decisions in such cells.

Many transformed cells retain the capacity to undergo terminal differentiation when treated with pharmacological agents belonging to one of several classes of differentiation-inducing drugs. Among these, hybrid polar compounds can induce differentiation in transformed cells derived from many tissues of all embryonic lineages [Marks et al., 1996]. The prototype of this class, hexamethylene bisacetamide (HMBA) has been extensively characterized in vitro [Marks et al., 1996] and has been clinically tested in patients with hematopoietic malignancies [Andreef et al., 1992]. The mechanism of action of HMBA has been studied extensively in murine erythroleukemia (MEL) cell lines. MEL cells are retrovirus-transformed hematopoietic precursors which are induced by hybrid polar agents to differentiate along the erythroid lineage [Marks et al., 1994; Marks et al., 1996]. Exposure to HMBA during the G_1 phase of the cell cycle causes the following G_1 to be prolonged. Thereafter, cell cycle rates return to normal, and at each cycle a fraction of the cells (~15%) are stochastically committed to terminal differentiation [Marks et al., 1994; Marks et al., 1996]. HMBA-induced biochemical changes that may be involved in prolonging G_1 include increased p21 levels, decreased activity of cdk4, and increased levels of dephosphorylated pRB [Kiyokawa et al., 1993; Marks et al., 1994; Kiyokawa et al., 1994; Zhuo et al., 1995; Marks et al., 1996]. The expression of c-myc and c-myb is also downregulated [Marks et al., 1996]. This G_1 lag is necessary, but not sufficient for commitment to terminal differentiation in response to HMBA [Kiyokawa et al., 1993]. Additional, still unidentified, biochemical events which occur during subsequent cycles are required to trigger terminal differentiation.

We investigated whether endogenously expressed notch-1 participates in cell fate decisions during HMBA-induced differentiation in MEL cells. Using various antisense strategies, we demonstrate that in these cells notch-1 is a necessary component of the decision switch among apoptosis, proliferation, and differentiation. In MEL cells, notch-1 prevents apoptosis, thus allowing the cells to progress through their differentiation program. This previously undescribed effect of notch-1 may further our understanding of the role of this receptor in modulating cell fate decisions and of the biological significance of notch-1 overexpression in neoplastic cells. Furthermore, these findings may indicate possible clinical uses of notch-1 antisense strategies.

MATERIALS AND METHODS

Cell Culture

Murine erythroleukemia Friend cells (MEL) from American Tissue Culture Collection, (TIB-55), were maintained in RPMI supplemented with 10% (v/v) heat inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 10^{-5} M β-mercaptoethanol (β-ME). To induce differentiation, logarithmically growing cells were plated at 1×10^5 cells/ml in medium containing 5 mM HMBA for 120 h. Cells were passaged to initial density at 72 h. Cells were assayed for the presence of hemoglobin [Orkin et al., 1975] by the addition of 1/10 volume of freshly prepared benzidine reagent (0.4% benzidine base, 2% hydrogen peroxide in 12% acetic acid). Benzidine positive cells were counted in modified Neubauer hemocytometers. Each sample was counted by two independent operators, blinded to the cell treatment, and readings were averaged.

RT-PCR

Total RNA was extracted from cells using Trizol reagent (Life Technologies, Bethesda,

MD). RT-PCR was performed using the Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin-Elmer, Oak Brook, IL) according to the manufacturer's instructions. Reactions were amplified in a Perkin-Elmer 2400 DNA thermal cycler for 40 cycles of denaturation at 94°C for 1 min, annealing, and extension at 65°C for 2 min. Amplification of mouse primers specific for notch-1, AATGGTCGAGGACCAGATGG (sense), and TTCAGGAGCACAACAGCAGC (antisense) generated a product of 431 bp. Primers specific for GAPDH, TCACCACCATGGAGAAGG (sense), and CAAAGTTGTCATGGATGACC (antisense) generated a 200 bp product. Negative and positive PCR controls were included in every experiment.

Notch-1 Antibody

A rabbit antiserum was raised against human notch-1 EGF repeats 11 and 12, a region that is highly conserved across species [Garces et al., 1997]. The antibody was shown to react with human and mouse notch-1 and Drosophila notch. In Western blots, this antiserum, but not the preimmune serum, recognized three immunoreactive bands: the notch-1 preprotein (~207 kDa), the extracellular cleavage product (180 kDa) corresponding to N^{EC} [Blaumueller et al., 1997] and present at the cell surface (C. Fuchs and L. Miele, unpublished data), and a 190–200 kDa band, that is probably a precursor to N^{EC}. These correlate with the expected notch-1 bands reported in the literature [Blaumueller et al., 1997].

Western Blotting

Cell pellets were solubilized in hot 2× SDS sample buffer containing 10% β-ME and analyzed by 4% SDS/PAGE. Proteins were electroblotted to Immobilon P (Millipore Corp., Bedford, MA) in 10 mM CAPS with 10% methanol at 0.75 A for 5 h. Protein bands were detected using a chemiluminescence Western blotting kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Antibodies to mouse hemoglobin and β-actin were from ICN (Irvine, CA) and Sigma (St. Louis, MO), respectively. Relative band intensities were determined by the Eagleview software (Stratagene, La Jolla, CA).

Antisense Oligonucleotides

Phosphorothioate oligonucleotides (S-oligos) were synthesized by the Core Facility at CBER. Sequences for the S-oligos were as follows: 1) EGF repeat region: sense GCTGTCTCAACGGTGGTACATGC, antisense GCATGTACCACCGTTGAGACAGC; 2) Lin/notch region: sense CCT-GGAAGAACTGCACGCAGTCT, antisense AGACTGCGTGCAGTTCTTCCAGG, scrambled GGACCTTCTTGACGTGCGTCAGA; 3) Ankyrin region: sense CAGCTTGCACAACCAGACAGACC, antisense GGTCTGTCTGGTTGTGCAAGCTG, scrambled TGCACGGTTCTGGTTGCGTGTGA. S-oligos corresponding to these regions of chick notch-1 have been used previously [Austin et al., 1995]. For S-oligo treatment, cells were induced to differentiate as described above, with the following modifications. Cells were plated in 96-well plates and S-oligos were added with the medium to a final concentration of 25 μM. Preliminary dose ranging experiments established this as the optimal concentration under our experimental conditions (data not shown). Medium and S-oligos were replaced on day 3. At least three independent batches of S-oligos were used in these experiments. A scrambled control S-oligo, to rule out artifactual effects due to base composition, was also included in some experiments and had similar effects to sense S-oligos. Comparison to the available sequences in Genbank indicated that sequences of the notch-1 antisense S-oligos are specific to notch-1.

Preparation of Notch-1 Antisense Plasmid

The pNotch-AS plasmid was generated by PCR amplification using cDNA fragments coding for mouse notch-1 (generous gift of Vijaya Manohar, CBER). Nucleotides +64–+1164 were amplified using Pfu DNA polymerase (Stratagene) and cloned in antisense orientation into the XhoI and NheI sites of pcDNA 3.1 (Invitrogen, La Jolla, CA). The primers used, including embedded restriction sites, were TTACTCGAGGCAGCTGGCGAGCAGGCATG (sense) and TTAGC-TAGCCGGACATTCGCAGTAGAAGG (antisense). The nucleotide sequence and orientation of the insert were confirmed by dideoxy sequencing using a Sequenase kit (Amersham, Arlington Heights, IL).

Transfection of MEL Cells

Logarithmically growing MEL cells (10^7) were pelleted, resuspended in 200 μl RPMI with 10% FCS and 20 μg of pNotch-AS plasmid or pcDNA3.1 vector plus 2 μg of pBABE to confer puromycin resistance [Morgenstern et al., 1990]. Cells were electroporated using a Bio-Rad gene

pulser (Richmond, CA) at 250 V and 960 μF. Cells were selected in the presence of 0.5 μg/ml puromycin (Sigma) and 700 μg/ml G418 (Life Technologies, Bethesda, MD) to increase the selective pressure for stable transfectants. Individual clones were isolated by limiting dilution.

Cell Growth Analysis of Notch-1 Transfected Clones

Cells were plated at 1×10^5 cells/ml (no HMBA) or 2×10^5 cells/ml (HMBA) in the same medium as parental MEL supplemented with 700 μg/ml G418. Cells were passaged at 72 h as described above. Cells were counted every 24 h for 120 h by hemocytometer.

Annexin V Binding Assay

Cells undergoing early apoptosis were identified by binding of Annexin-V to membrane phosphatidylserine and assayed using FITC-conjugated Annexin-V (Pharmingen) according to the manufacturer's instructions. Briefly, 1×10^6 cells were washed twice in PBS, and resuspended at 1×10^6 cells/ml in binding buffer (Pharmingen, San Diego, CA). Propidium iodide (PI, final concentration 5 μg/ml) and Annexin-V (5 μl) were added to 1×10^5 cells, incubated for 15 min in the dark at room temperature, and analyzed by flow cytometry using a Becton Dickinson FACScan instrument with CellQuest software.

Cell Cycle and Apoptosis Analysis by PI Staining

For cell cycle analysis, cells were synchronized by density arrest [Ryan et al., 1993], then plated at 1×10^5 cells/ml in medium containing 5 mM HMBA. For transfected clones, 700 μg/ml G418 was added to the medium. At 16 h, cells were harvested, fixed in 1% paraformaldehyde for 15 min, and then 70% ethanol overnight. Cells were resuspended, at constant cell density, in PI solution (50 μg/ml PI, 0.1% Triton-X-100, 200 μg/ml RNAase A) for 1 h at room temperature with mixing. DNA content was determined using a Becton-Dickinson FACScan flow cytometer. In cell cycle analyses, apoptotic and dead cells were gated out and only the viable population was analyzed. In apoptosis experiments with S-oligos, unsynchronized cells were plated in 96-well plates with HMBA, with or without S-oligos (25 μM). DNA contents were analyzed as described above at 120 h. Cells in the sub-G_1 peak were scored as apoptotic [Sherwood et al., 1995].

Statistical Analyses

Multiple treatments in S-oligo experiments were compared by one-way ANOVA with Bonferroni correction for multiple comparisons. Unpaired, two-tailed *t*-tests ($\alpha = 0.05$) were used for all other comparisons.

RESULTS

Notch-1 mRNA and Protein Levels are Modulated Differently in MEL Cells During Growth and HMBA-Induced Differentiation

To determine whether notch-1 expression is regulated during HMBA-induced MEL cell differentiation, notch-1 mRNA levels were analyzed in MEL cells plated at equal density and maintained in culture for 4, 8, 24, or 120 h in the absence or presence of HMBA. A representative time course of notch-1 mRNA levels, determined by RT-PCR analysis, is shown in Figure 1A. Notch-1 mRNA was observed in MEL cells induced with HMBA for 4 h; but decreased to undetectable levels in the continued presence of HMBA. In contrast, notch-1 mRNA was undetectable in control cells at 4 and 8 h, while significant levels were observed at 24 and 120 h. GAPDH mRNA was present in both uninduced and induced MEL cells throughout the 120 h.

Notch-1 steady state protein levels were determined by Western blot using an antibody recognizing an extracellular epitope of notch-1 [Garces et al., 1997] (Fig. 1B). Three immunoreactive bands were detected: the notch-1 preprotein (~207 kDa), the extracellular cleavage product (180 kDa) corresponding to mature N^{EC} [Blaumueller et al., 1997] and a 190–200 kDa band, probably a precursor to N^{EC}. Notch-1 protein levels reflected the notch-1 mRNA pattern of expression. In the presence of HMBA, notch-1 protein was reproducibly evident in the early stages of induction, but became essentially undetectable at 120 h. Significant amounts of hemoglobin were detected at this time, indicating that erythroid differentiation was taking place and that the decline in notch-1 levels was not the consequence of a generalized drop in protein synthesis. In uninduced MEL cells, notch-1 protein was present at low levels at 4 h; these levels increased by 24 h and were maintained throughout the 120 h. In 24–20 h time course experiments, notch-1 protein was markedly decreased in HMBA-treated cells at 72 h (<4% of control), and became undetectable at 120 h. β-actin was slightly lower in all HMBA-treated cells throughout the time course, and

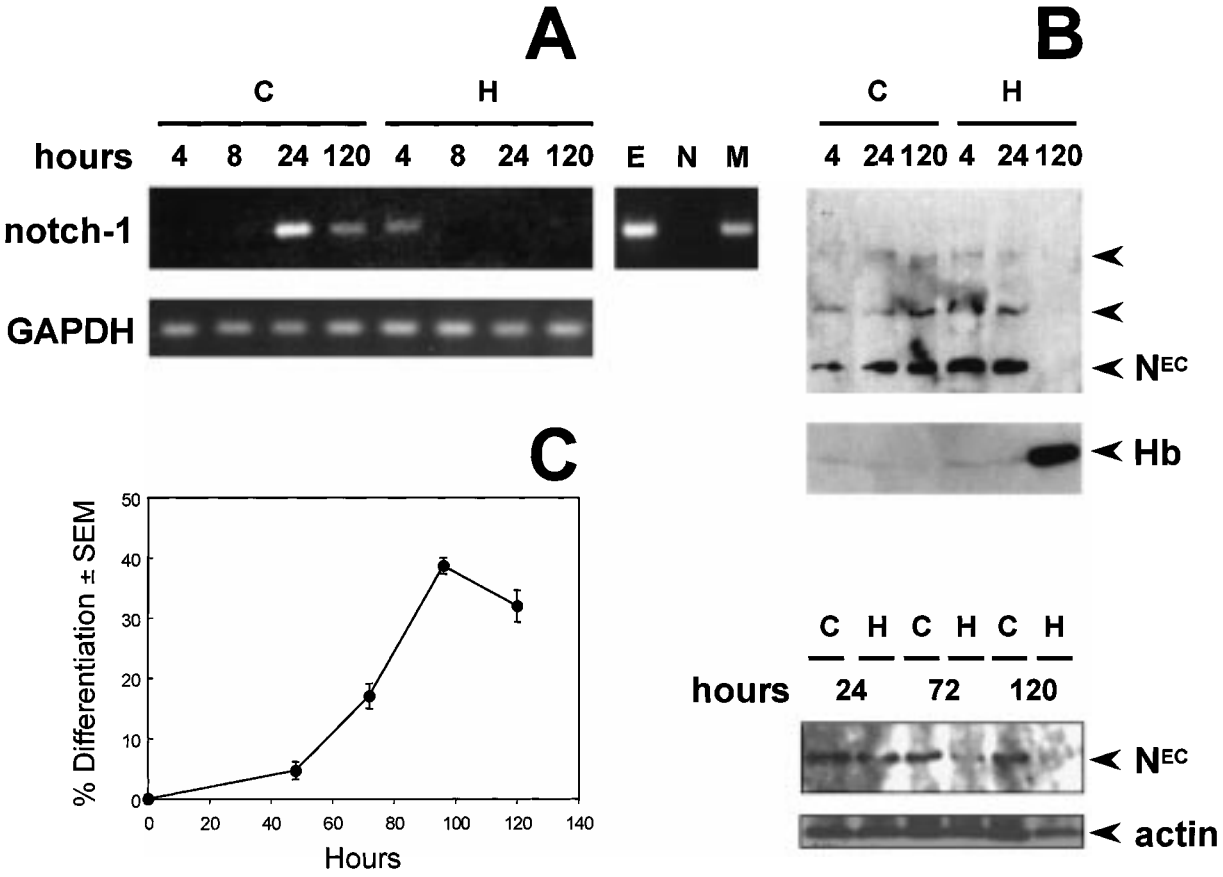

Fig. 1. HMBA modulates the expression of notch-1 mRNA and protein in MEL cells. **A:** Notch-1 mRNA levels were analyzed in MEL cells plated at equal density and maintained in culture for 4, 8, 24, or 120 h in the absence (C) or presence (H) of HMBA. Total RNA was isolated and analyzed by RT-PCR to determine steady-state levels of notch-1 and GAPDH mRNA. PCR negative and positive controls were included in every experiment. E, 16-day mouse embryo cDNA; N, no DNA; M, mouse cell line 70Z/3. **B:** Western blot analysis of notch-1, globin and β-actin protein levels. Top panel: MEL cells were maintained for 4, 24, or 120 h, in the absence (C) or presence (H) of HMBA. Three immunoreactive bands (designated by arrows) are recognized by the notch-1 antibody: the notch-1 pre-protein (~ 207 kDa), a putative N^{EC} precursor band (190–200 kDa) and the extracellular subunit N^{EC} (180 kDa). Globin (Hb) protein (second panel) was abundantly expressed in HMBA induced MEL at 120 h indicating that differentiation was in progress. A 24–120 h time course experiment (third panel) showed that notch-1 level was markedly reduced (< 4% of control) in HMBA-treated cells (H) compared to control (C) at 72 hours and essentially undetectable at 120 h. β-actin was somewhat lower in HMBA-treated cells throughout the time course (between 35 and 50%), and showed some decline at 120 h (approximately 50% compared to 24 h; bottom panel). **C:** A representative time course of accumulation of benzidine-positive cells in MEL cells induced with HMBA. At the indicated times, MEL cells were removed and stained with benzidine to assess differentiation.

showed a modest decline at 120 h in HMBA-treated cells, presumably due to the structural and cytoskeletal changes associated with terminal differentiation [Marks et al., 1996]. These data suggest that HMBA treatment causes an early upregulation in notch-1 levels, followed by a rapid, progressive decline. Differentiation is accompanied by disappearance of notch-1, a dramatic increase in globin and a modest decrease in β-actin. These patterns of notch-1 mRNA and protein expression were observed reproducibly in at least four different experiments.

During the 120 h of culture with HMBA (Fig. 1C), differentiated cells increased steadily from 48 h onwards, generally reaching 35–45%, at 120 h. A slight drop in the number of differentiated cells was observed in some experiments at 120 h, possibly reflecting a balance between death of previously differentiated cells and differentiation of new cells. We did not continue cultures beyond 120 h, since at this time notch-1 was reproducibly undetectable. Expression of notch-1 was not restored by passaging HMBA-treated cells in HMBA-free medium at 120 h (not shown). This suggests that commitment to

terminal differentiation is accompanied by irreversible loss of notch-1 expression.

Notch-1 Antisense S-Oligos Inhibit HMBA-Induced MEL Cell Differentiation and Increase Cell Death

To determine if notch-1 plays a role in HMBA-induced differentiation of MEL cells, notch-1 antisense S-oligos were used to deregulate expression of the protein. Three sets of 23-mer S-oligos (sense and antisense) were synthesized corresponding to the EGF repeat, the Lin-12 and the ankyrin regions of mouse notch-1. S-oligos and HMBA were added to the medium at time 0, and cells were maintained in culture for 120 h. The percentage of benzidine-positive cells at 120 h was reproducibly decreased by approximately half in the presence of antisense compared to sense notch-1 S-oligos (Fig. 2A). Significant inhibition ($P < 0.02$) compared to sense controls was seen with each of the three notch-1 antisense S-oligos. When antisense S-oligos were added at 72 h, differentiation was not inhibited (not shown).

To determine whether the decrease in differentiation was accompanied by an increase in apoptosis, the late apoptotic fraction (sub-G_1 peak) was determined by flow cytometry in cells treated with Lin-12 sense, antisense, and scrambled S-oligos in the presence of HMBA

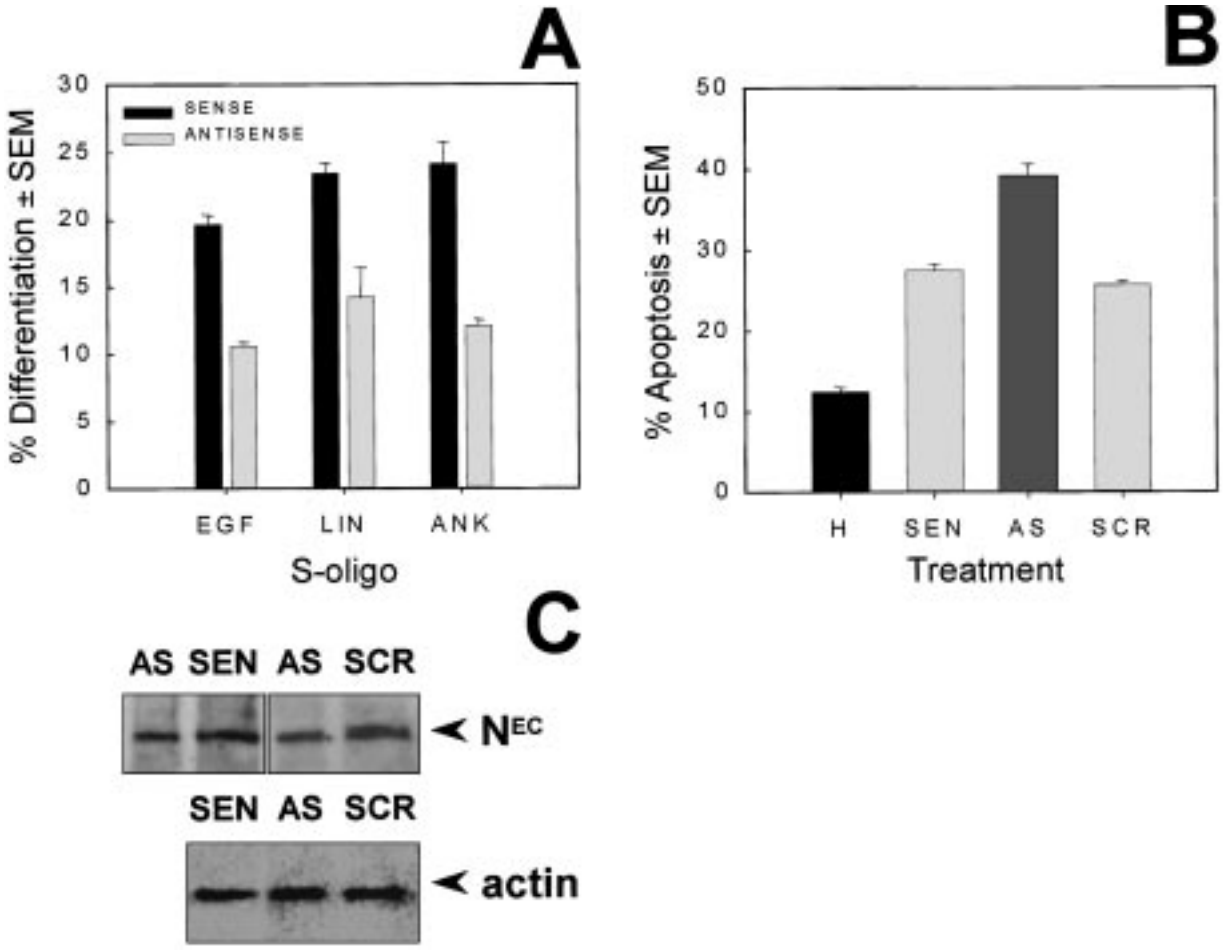

Fig. 2. Effects of notch-1 S-oligos on differentiation and apoptosis in HMBA-induced MEL cells. **A:** Three sets of S-oligos (sense and antisense) were synthesized corresponding to the EGF repeat (EGF), the Lin-12 (LIN) or the ankyrin (ANK) region. Either sense or antisense S-oligos (25 μM) were added to MEL cells together with HMBA and maintained in culture for 120 h. Differentiation was scored at 120 h. **B:** MEL cells maintained in the presence of HMBA alone (H) and sense (SEN), antisense (AS) or scrambled (SCR) LIN-12 notch-1 S-oligos for 120 h, were fixed and stained with PI. The fractions of apoptotic (sub-G_1 DNA content) and viable cells were determined by flow cytometry. **C:** MEL cells treated with HMBA plus sense (SEN), antisense (AS) or scrambled (SCR) LIN-12 notch-1 S-oligos for 48 h were analyzed by Western blotting for notch-1 and β-actin. In three separate experiments, two of which are shown here, the AS S-oligo reduced notch-1 levels at least 50% compared to either SEN or SCR. No specific decrease in β-actin levels was observed with the AS oligo compared to either SEN or SCR.

(Fig. 2B). While all S-oligos increased the apoptotic fraction compared to HMBA alone, the antisense S-oligo had a significantly larger effect than either sense or scrambled controls ($P < 0.001$). A parallel decrease in viability (cells within the G1, G2/S, and M regions) was observed.

The Lin-12 antisense S-oligo, but not sense or scrambled controls, decreased expression of notch-1 protein by at least 50% at 48 h in three experiments (Fig. 2C). No difference in β-actin levels was observed in cells treated with sense, antisense, and scrambled Lin-12 S-oligos.

Taken together, these results strongly suggest that notch-1 is necessary for HMBA induced differentiation in MEL cells and that decreases in notch-1 expression induced by a specific antisense effect result in decreased differentiation and increased apoptosis in these cells.

Downregulation of Notch-1 Expression in Stably Transfected Notch-1 Antisense MEL Clones Inhibits Differentiation and Growth in the Presence of HMBA

Further analyses with S-oligos were limited by their non-specific toxicity and the need to conduct experiments in small scale. Additionally, the effects of HMBA on cell permeability [Marks et al., 1996] made a comparison of S-oligos effects in the presence and absence of HMBA virtually uninterpretable. Therefore, we generated several MEL clones stably transfected with an 1100 bp antisense notch-1 construct (n1-AS) or with vector alone. The antisense construct encompasses the 9 N-terminal EGF repeats of the notch-1 preprotein. This region is less conserved among notch family members than the rest of the extracellular subunit, and does not include the sequences targeted by the antisense S-oligos. In all the n1-AS clones used for further experiments, the basal levels of notch-1 protein, but not those of β-actin, were reduced by at least 50% in logarithmically growing cells compared to vector-transfected clones (Fig. 3A).

Notch-1 expression time course experiments were performed with four notch-1 AS clones and three vector-transfected clones. Representative Western blots from two clones are shown in Figure 3A. Notch-1 protein levels in vector-transfected clones reflected the pattern seen in parental MEL. The decline in notch-1 levels in HMBA was somewhat slower, with protein still detectable at 72 h and almost undetectable at 120 h, possibly due to slower overall growth in the presence of G418. In n1-AS clones, the decrease in notch-1 induced by HMBA was accelerated. Levels of notch-1 protein in n1-AS clones were lower at 24 h compared to vector-transfected controls and became essentially undetectable by 72 h. In the absence of HMBA, vector-transfected clones exhibited a comparable pattern of notch-1 expression to parental MEL cells under similar conditions (Fig. 3A). In n1-AS clones, notch-1 protein was detectable at lower levels than in vector-transfected clones at 24 h and was barely detectable at 72 and 120 h.

Differentiation time course experiments were performed with four independent n1-AS clones and three vector-transfected clones. HMBA-induced differentiation was strongly inhibited in n1-AS clones compared to vector transfected clones (Fig. 3B). Significant differences ($P < 0.05$) were observed from 48 h onwards, and became very pronounced at 72 h. The percentage of benzidine-positive cells in the AS clones reached approximately 6% and did not increase further. In contrast, in vector-transfected clones benzidine-positive cells increased linearly reaching approximately 30% at 120 h.

In MEL cells, proliferation and differentiation are tightly related. Thus, we analyzed the growth kinetics of transfected MEL clones in the presence or absence of HMBA (Fig. 3C,D). In these experiments, total cell numbers were determined, without viability corrections. In the presence of HMBA, vector transfected clones showed slow but continued growth after 24 h, which paralleled their differentiation kinetics, as observed in parental MEL cells [Fibach et al., 1977]. In contrast, n1-AS clones showed no significant growth after 48 h ($P < 0.05$ compared to vector from 48 h onwards). In the absence of HMBA both vector and n1-AS-transfected clones grew more rapidly (average doubling time was approximately 3.5 times shorter than vector-transfected clones in HMBA), and there was no significant difference between the growth rates of n1-AS and vector-transfected clones (Fig. 3D). This indicates that the differences observed in the presence of HMBA are not due to an inherently slower proliferation rate of n1-AS clones.

Downregulation of Notch-1 Expression in Stably Transfected Notch-1 Antisense MEL Clones Increases Apoptosis in the Presence or Absence of HMBA

Experiments using S-oligos suggested that treatment of MEL cells in the presence of HMBA with antisense as opposed to sense notch-1 S-oligos brought about an increase in apoptosis

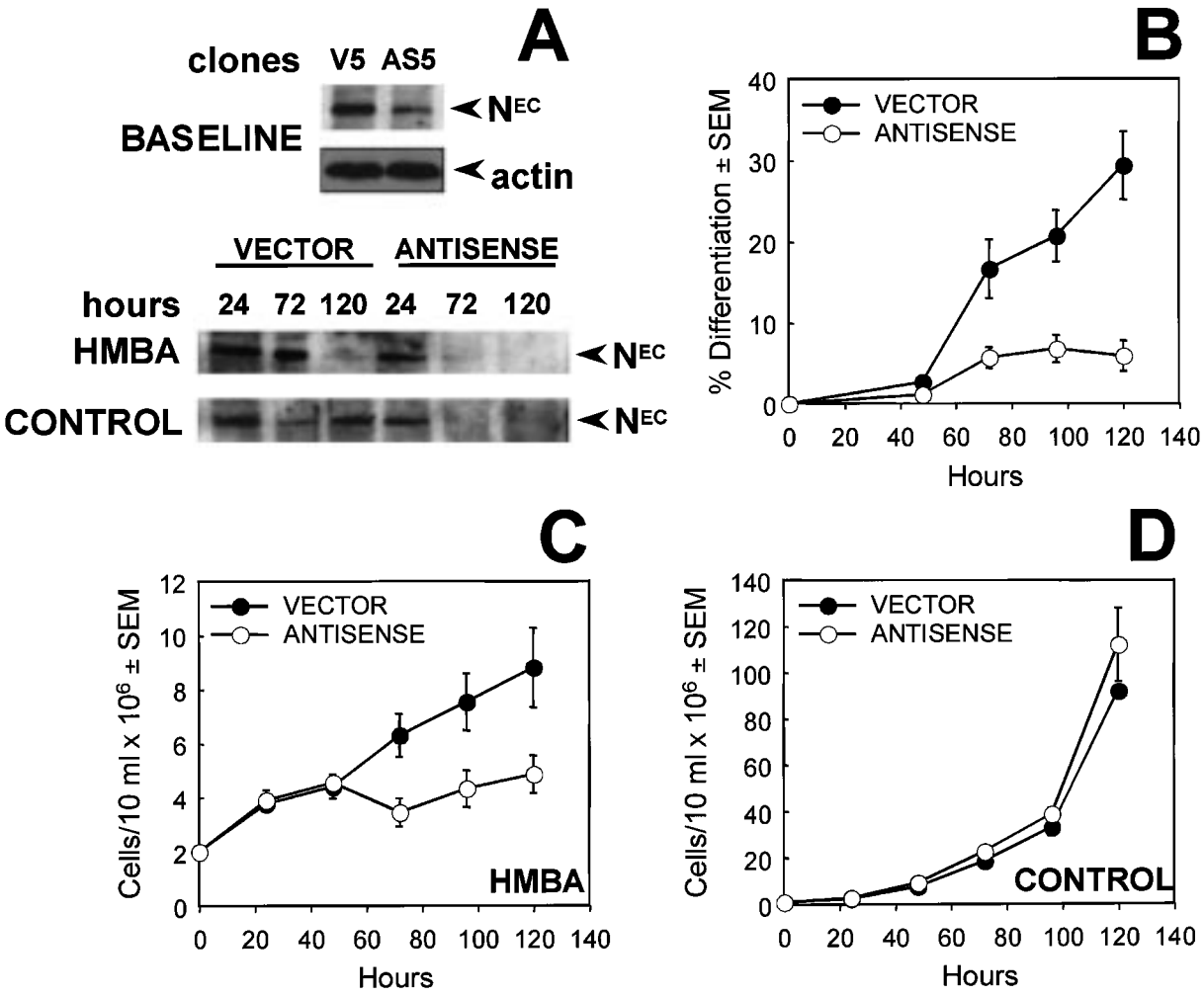

Fig. 3. Notch-1 expression, differentiation, and growth kinetics of n1-AS and vector-transfected MEL clones. **A:** Equal amounts of protein (50 μg) isolated from cell extracts of n1-AS and vector transfected MEL clones were analyzed by Western blot. Results for representative n1-AS (AS5) and vector (V5) clones are shown for notch-1 (top panel) and β-actin (second panel). Expression of the notch-1 protein in n1-AS cells was reduced by at least 50% compared to vector-transfected control. No difference in β-actin levels was observed. The third and fourth panels show a Western blot analysis of notch-1 in V5 (VECTOR) and AS5 (ANTISENSE) clones maintained for 24, 72, or 120 h in the presence or absence of HMBA. The notch-1 extracellular band N^{EC} is shown. **B:** Time course of accumulation of benzidine positive cells in MEL transfected clones. Cell lines were maintained in the presence of HMBA for 120 h. At the indicated times, cells were removed, stained with benzidine, and scored for differentiation. Growth kinetics of n1-AS and vector transfected clones with or without HMBA are shown in C and D. Transfected cells were plated in the presence **(C)** or absence **(D)** of HMBA and maintained in culture for 120 h. Cells were counted every 24 h by hemocytometer. In each experiment, four n1-AS clones (ANTISENSE) and three vector-transfected clones (VECTOR) were analyzed.

(see Fig. 2B). N1-AS clones showed a strong inhibition of both growth and differentiation from 48 h onwards. Thus, we investigated whether the observed effect of N1-AS may be due to cells switching from a growth and differentiation program to an apoptosis program. To test this hypothesis, apoptosis and viability in transfected clones were analyzed by flow cytometry with annexin V and PI. Early apoptotic cells, (annexin V positive, PI negative), were quantitated every 24 h for 120 h in the presence or absence of HMBA. In the presence of HMBA, the fraction of early apoptotic cells in both vector and n1-AS transfected clones increased gradually after 24 h. However, the apoptotic fraction was significantly higher ($P < 0.05$) in n1-AS clones from 48 h onwards (Fig. 4A), reaching approximately 70% at 120 h. The apoptotic fraction increased dramatically from 24 to 48 h in the n1-AS clones compared to vector-transfected cells. This was accompanied by a striking decrease in viability (PI negative, annexin negative cells) in all n1-AS clones (Fig. 4B). Viability decreased steadily in all clones after 48 h in the presence of HMBA in all clones. However, n1-AS clones showed an earlier and much larger drop

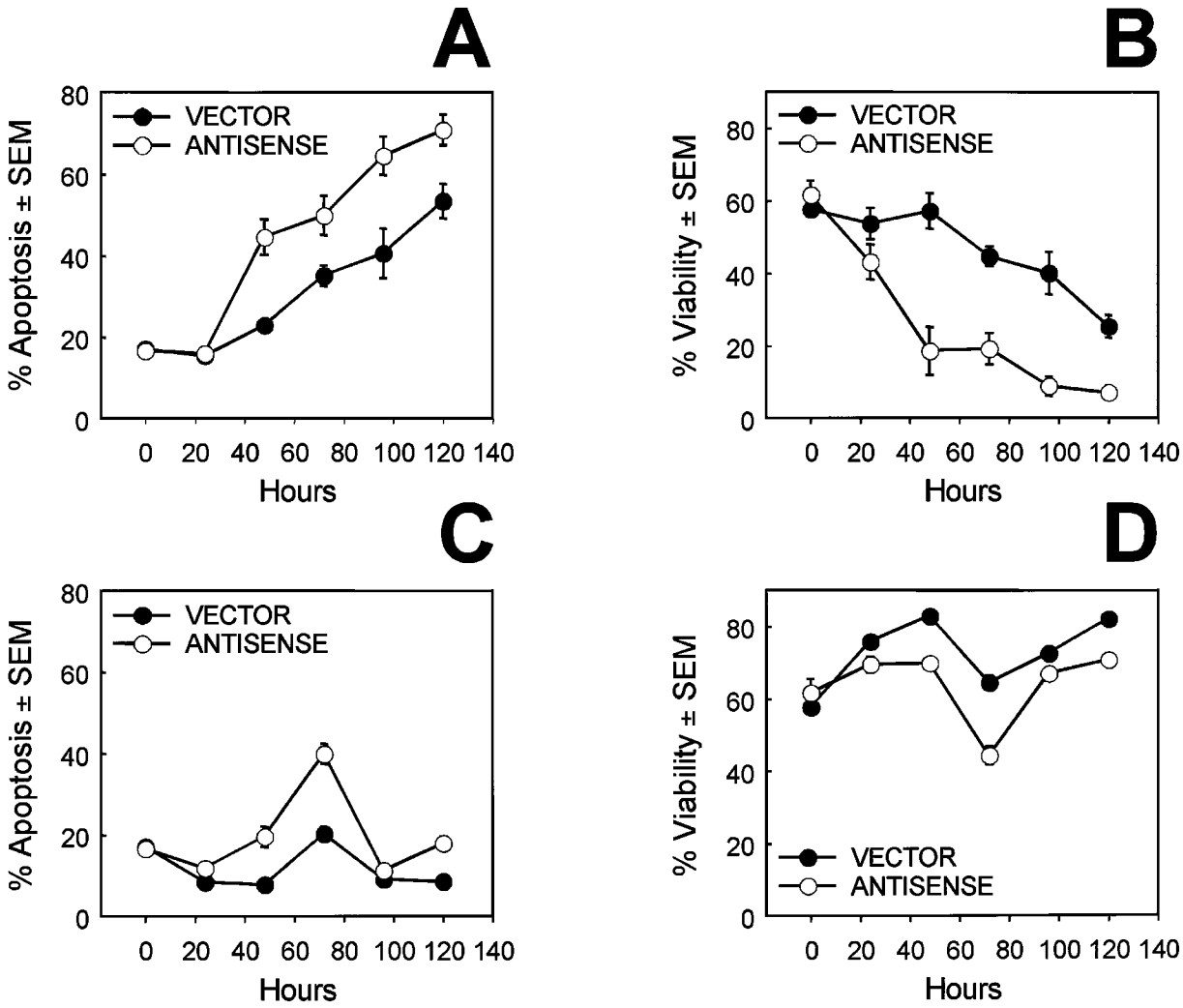

Fig. 4. Apoptosis and viability in n1-AS and vector-transfected clones with or without HMBA. Cell lines were maintained in the presence **(A,B)** or absence **(C,D)** of HMBA for 120 h. At the indicated times, cells were removed, treated with FITC-conjugated Annexin V and PI and analyzed by flow cytometry. 10^4 cells per point were analyzed for each clone. Annexin V-positive, PI-negative cells were scored as undergoing apoptosis. Viable cells were identified as the Annexin V-negative, PI-negative population. Results are expressed as the mean ± SEM of the n1-AS transfected clones (n = 4) or the vector clones (n = 3) and are representative of three similar experiments conducted with all clones.

in viability than vector-transfected clones (P at least < 0.05 for all time points from 48 h onwards). At 48 h, more than 80% of the cells in all n1-AS clones were either entering apoptosis (Fig. 4A) or dead (Fig. 4B), with an average viability of 17%. At the same time, vector-transfected clones showed no appreciable change in viability and only a minor increase in apoptosis compared to time 0. At 120 h, the average viability of n1-AS clones was less than 7%, with one clone declining to essentially 0, compared to approximately 30% in vector-transfected clones. These data indicate that the arrest in cell growth and differentiation observed from 48 h onwards is most likely the result of an increased number of cells entering an apoptotic program in n1-AS clones.

Spontaneous apoptosis rates in the absence of HMBA showed a pattern roughly parallel to cell density, increasing up to 72 h. At this time, when cells were passaged into fresh medium at initial density (Fig. 4C), apoptosis rates dropped. This pattern may be due to growth factor, nutrient, or oxygen deprivation in densely growing cultures. The early apoptotic fraction in n1-AS clones was significantly higher than in vector-transfected cells (P at least < 0.02 after 24 h) throughout the time course, reaching approximately 40% at 72 h, immediately before cells were passaged. Higher apoptotic fractions in AS clones were paralleled by significantly lower viability (P at least < 0.05, Fig. 4D).

These results suggest that decreases in notch-1 expression in MEL cells are associated

with increased levels of apoptosis, regardless of the presence of HMBA. However, in the absence of HMBA, passaging into fresh medium at 72 h appeared to rescue early apoptotic n1-AS cells, since the overall growth curves were not significantly different.

The Early G_1 Lag Induced by HMBA is Not Affected in Notch-1 Antisense MEL Clones

Treatment of MEL cells with HMBA induces a G_1 lag in the cell cycle immediately subsequent to the first G_1 in the presence of the inducer [Marks et al., 1996; Marks et al., 1994; Kiyokawa et al., 1993; Zhuo et al., 1995]. This prolonged G_1 is necessary for later commitment to differentiation [Kiyokawa et al., 1993]. Therefore, we analyzed cell cycle distribution in n1-AS and vector-transfected MEL clones. In these experiments, cells were synchronized in G_0/G_1 by density arrest and released from synchronization in HMBA-supplemented medium. DNA content in viable cells was determined at 16 h, the optimal time to detect a G_1 lag in our system. No significant differences in cell cycle distribution were observed between n1-AS and vector-transfected clones. G_1 fractions were $52.4 \pm 0.8\%$ for n1-AS cells vs. $53.2 \pm 1.4\%$ for vector-transfected cells. G_2/S (26.5 ± 1.3 vs. 24.4 ± 0.8) and M fractions (21.8 ± 1.9 vs. 22.9 ± 2.1) were also not significantly different. Similar results were observed in MEL cells treated with S-oligos (not shown). This indicates that the effect of notch-1 on MEL cells apoptosis is not mediated by inhibition or enhancement of the HMBA-induced G_1 lag.

DISCUSSION

We investigated the role of endogenous notch-1 expression in cell fate determination in MEL cells, and identified a novel function of notch-1: protection from apoptotic cell death. In MEL cells treated with HMBA, notch-1 appears to allow the completion of the differentiation program by preventing premature apoptosis. Notch-1 also appears to protect MEL cells from spontaneously occurring apoptosis when cell density increases. This anti-apoptotic effect of notch-1 may provide a survival advantage to transformed cells overexpressing notch-1. During pharmacologically induced differentiation, notch-1 appears to be upregulated early after HMBA exposure, while commitment to terminal differentiation is associated with irreversible disappearance of notch-1. When notch-1 levels were prematurely downregulated during HMBA treatment, by two different antisense strategies targeting four different regions in the notch-1 gene, increased apoptosis and decreased differentiation ensued. Spontaneous apoptosis rates in the absence of differentiation inducers were also higher in n1-AS cells. This suggests that the level of notch-1 expression controls apoptosis susceptibility in MEL cells.

We used antisense approaches to study the role of full length, endogenously expressed notch-1 in this model rather than enforced expression of truncated, constitutively activated forms of notch-1. Such constructs are useful in addressing mechanistic questions. However, truncated forms of notch-1 are known to have biological activities not displayed by the intact protein [Capobianco et al., 1997]. These proteins often localize to the nucleus [Capobianco et al., 1997] in amounts far larger than needed to mediate notch signaling [Schroeter et al., 1998; Struhl et al., 1998] and can interact with other ankyrin repeat-containing molecules [Guan et al., 1996] which may not normally participate in notch signaling. Thus, a novel biological effect observed by overexpressing constitutively active notch-1 in a cell line which already expresses full length notch-1 may not necessarily be indicative of the function of the intact protein.

We hypothesize that notch-1 controls the apoptosis threshold in cycling MEL cells. During HMBA-induced differentiation, notch-1 prevents premature apoptosis of precommitted cells, thus allowing them to continue replicating and reach critical levels of intracellular mediator(s) that result in recruitment to commitment [Marks et al., 1996]. Whether notch-1 has a direct effect on differentiation in MEL cells cannot be determined from these studies. Committed cells appear to irreversibly lose notch-1 expression. It is tempting to speculate that notch-1 downregulation may signal commitment to terminal differentiation in MEL cells. If this signal is received prematurely or inappropriately, when a differentiation program cannot be completed, the cells may undergo apoptosis, a common fate of many terminally differentiated cells. Interestingly, in multiple myeloma cells, which represent a highly differentiated B cell phenotype, HMBA induces apoptosis [Siegel et al., 1998].

It is possible that apoptosis occurring during HMBA treatment in n1-AS MEL cells may represent an abortive attempt to differentiate towards a different phenotype. However, n1-AS cells also have higher rates of spontaneous apoptosis and significantly lower viability than con-

trols as early as 24 h after culture in HMBA-free medium. This suggests that in MEL cells notch-1 may modulate common step(s) in the decision switch between apoptosis, differentiation, and growth rather than a signaling pathway activated during HMBA-induced differentiation. As both p53 alleles are mutant in MEL cells [Ben-David et al., 1991], apoptosis in n1-AS MEL clones is likely to occur by p53 independent pathways. Our data rule out prolongation or inhibition of the G_1 lag as possible mechanisms for the effects of notch-1 antisense. Which notch-1 ligand, if any, mediates the anti-apoptotic effect of notch-1 in MEL cells remains unclear. We were unable to detect expression of Delta-like gene 1 [Bettenhausen et al., 1995] in MEL cells by RT-PCR (not shown). However, this does not rule out other, as yet uncharacterized, murine ligands.

The mechanisms of notch-1's anti-apoptotic activity in MEL cells are currently under investigation. Potential targets of notch signaling which are involved in apoptosis control include NF-κB/rel family proteins and the ras-JNK pathway. Notch-1 has been recently shown to regulate the NF-κB2 promoter through CBF-1 [Oswald et al., 1998]. Additionally, there is indirect evidence that JNK-mediated activation of E47 is regulated by notch through a CBF-1 independent mechanism [Ordentlich et al., 1998]. A possible additional target is Bcl-2, which is downregulated by HMBA in other cells [Siegel et al., 1998]. Our findings raise the question whether the anti-apoptotic effect of notch-1 is part of its physiological role, at least in some instances, or is a function of notch-1 overexpression occurring in transformed cells. Several human tumors [Zagouras et al., 1995; Daniel et al., 1997] and tumor cell lines [Aster et al., 1994] (C. Fuchs and L. Miele, unpublished data) express large amounts of apparently full-length notch-1 protein. Our results indicate that in MEL cells, decreased expression of notch-1 is associated with increased sensitivity to apoptosis. In non-transformed cells of various lineages [Austin et al., 1995; Garces et al., 1997] notch-1 antisense strategies affect differentiation but have not been shown to cause apoptosis. While it is possible that the anti-apoptotic effect of notch-1 may be cell-type specific, it is tempting to speculate that overexpression of notch-1 may have a survival value for some transformed cells. In naturally occurring tumors, resistance to apoptosis generally increases due to mutations which accumulate during tumor progression and after treatment. Overexpression of notch-1 may be one of the mechanisms by which tumor cells gain increased resistance to apoptosis. If so, downregulating notch-1 expression may have potential therapeutic uses in tumors overexpressing notch-1. Since apoptosis promoted by antisense notch-1 does not appear to require p53, such strategies may be used in p53-null tumors. These include over 50% of human malignancies [Hollstein et al., 1991]. The possible use of antisense notch-1 oligonucleotides or gene therapy strategies with differentiation inducers or other antineoplastic treatments to enhance the rate of apoptosis in human malignancies deserves further investigation.

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