Down-Regulation of Notch1 Expression is Involved in HL-60 Cell Growth Inhibition Induced by 4-Hydroxynonenal, a Product of Lipid Peroxidation

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Abstract: The role of the Notch1 pathway has been well assessed in leukemia. *Notch1* mutations are the most common ones in T acute lymphoblastic leukaemia patients which carry either oncogenic Notch1 forms or ineffective ubiquitin ligase implicated in Notch1 turnover. Abnormalities in the Notch1-Jagged1 system have been reported also in acute myelogenous leukaemia (AML) patients where Jagged1 is frequently over-expressed. Moreover, activating Notch1 mutations, as well, can occur in human AML and in leukemia cases with lineage infidelity. As a result, Notch1 signalling inhibition is an attractive goal in leukaemia therapy. Blockage/delay in cell differentiation and/or increase of proliferation are the main results of Notch1 signalling activation in several leukemic cell lines. Moreover, specific genes involved in cell growth control have been identified as Notch1 transcriptional targets, i.e. Cyclin D1 and c-Myc.

4-Hydroxynonenal (HNE), an aldehyde produced during lipid peroxidation, is involved in several pathological and physiological conditions, including inflammation; atherosclerosis; and neurodegenerative and chronic liver diseases. Moreover HNE has an antiproliferative/ differentiative effect in several cell lines, by affecting the expression of key genes, such as oncogenes (e.g. c-Myc, c-Myb), cyclins and telomerase.

This prompted us to study the effect of HNE on Notch1 expression and its related signalling in HL-60 cells, a leukemic cell line widely used for differentiation studies. RT-PCR as well as Western blot assay showed Notch1down-regulation in HNE-treated HL-60 cells. The expression of Hes1, a Notch1 target gene, was concomitantly down-regulated by HNE treatment, reflecting Notch1 signalling inhibition.

DAPT, an inhibitor of Notch activity, when added contemporary to HNE, further increased cell growth inhibition, without affecting apoptosis. Moreover, DAPT treatment reversed the HNE-induced differentiation. Overall these results suggest that Notch1 is a target for HNE and its down regulation is a key event in HNE-mediated inhibition of cell proliferation in the HL-60 cell line. By contrast our data do not support a role for Notch1 in HNE- induced differentiation or apoptosis.

Key Words: Notch1, Hes1, HNE, HL-60 leukemic cells, proliferation, differentiation.

INTRODUCTION

Notch is a transmembrane receptor that regulates developmental processes and cell-fate decisions. Notch activation occurs through its binding to the ligands of the Delta/Serrate/Lag-2 (DSL) family, which results in two proteolytic cleavages catalysed by the ADAM-family metalloproteases [1] and presenilin, an enzyme complex containing γ-Secretase [2]. The second cleavage releases the Notch intracellular (IC) domain into the nucleus where it promotes transcription of specific target genes [3].

The effects of Notch on individual cells are highly dependent on signal dose and context, and include increased survival or death, proliferation or growth arrest, and commitment to or blockage of differentiation.

The Notch pathway deregulation exerts an oncogenic effect in many lineages of the hematopoietic compartment [4-6] and in different solid tumors [7,8]. Human Notch1 was discovered as a partner gene in t(7;9)(q34; q34.3) chromosomal translocation in T acute lymphoblastic leukemia (T-ALL) patients [9]. Notch1 mutations are the most common ones in T-ALL, they have been identified in approximately 70% of T-ALL and cause ligand-independent signalling and/or prolong Notch1 half-life in the nucleus [10-12]. Abnormalities in the Notch1 and Jagged1 (member of DSL family) systems are reported also in acute myelogenous leukaemia (AML) patients where Jagged1 is significantly over-expressed compared with other forms of acute leukaemia [5]. In AML patients the frequent translocations t(15; 17), t(8;21) and t(11;17) produce respectively the PML/RAR, AML1/ETO and PLZF/RAR fusion proteins which cause over-expression of the Notch ligand Jagged1 [13]. Activating Notch1 mutations can rarely occur in human AML, as well. Recently Fu and collaborators [14] reported one primary AML case carrying a missense mutation, Pro2439Leu (7316C/T), found in the PEST domain. The authors suggest that this mutation is not a single nucleotide polymorphism since it was not detected after complete remission. Furthermore, activating mutations in Notch1 have been found in a single primary AML sample, in three out of 23 AML cell lines and

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in the diagnostic (myeloid) and relapsed (T-lymphoid) clones of a patient with lineage switch leukaemia [15].

Activation of Notch1 signalling results in delay or blockage of cell differentiation induced by common compounds, in several leukemic cell lines [16-18] and in normal haematological progenitors [19]. Although signals mediated through Notch receptors have diverse outcomes, only a fairly limited set of Notch target genes have been identified in various cellular/developmental contexts. The hairy/enhancer of split (Hes) genes are highly conserved target genes that are regulated by Notch in multiple cell types [20]. Notch interferes with the cell cycle machinery by down-regulating expression or activity of cell cycle inhibitors, such as $p21^{Cip1}$ [21], p53 activity [22] and $p27^{Kip1}$ [23]. Moreover, specific genes, involved in cell growth control, were identified as a direct transcriptional target of Notch1, such as Cyclin D1 [24] and c-Myc [25]. Given the ability of Notch to regulate cell cycle progression and apoptosis, it is not surprising that several compounds which affect cell proliferation and viability, i.e. hexamethylene bisacetamide (HMBA) and resveratrol, also produce changes in Notch signalling in several cell lines from haematological malignancies [26,27].

4-Hydroxynonenal (HNE), an aldehyde, produced from lipid peroxidation, is capable of several biological effects [28]. For many years the role of HNE has been studied in cell damage as the ultimate mediator of toxic effects elicited by oxidative stress. Now it is considered as a bioactive molecule able to affect signal transduction, gene expression, cell proliferation, and, more generally, the response of the target cells [29]. Moreover, HNE has been detected "*in vivo*" in several pathological conditions, including inflammation, atherosclerosis, chronic degenerative diseases of the nervous system and chronic liver diseases [30].

HNE can influence tumor cell growth, apoptosis and differentiation, by affecting expression of specific genes [29, 31]. Low levels of lipid peroxidation occurring in tumor cells make HNE production often undetectable. Nonetheless, HNE, at a concentration similar to that found in normal tissues (1 μM), is able to inhibit cell proliferation and occasionally to reactivate the differentiation program [32, 33]. Accordingly, HNE regulates the expression of genes involved in cell proliferation control, i.e. the oncogenes c-Myc and c-Myb [34-37], some cyclins (Cyclin D1, D2, A) [38] and the E2F4 transcription factor [39]. The role of HNE in controlling the replicative potential of cells was recently extended to its ability to inhibit telomerase activity and hTERT expression in HL-60, U-937 and ML-1 leukemic cell lines [37].

In the view of HNE ability to affect cell proliferation and regulate genes involved in cell cycle control, we postulated that HNE could also influence Notch- 1 expression.

In this study we investigated the effect of HNE on the Notch1 pathway in the HL-60 human leukemic cell line. Moreover, the possible synergistic effect of HNE with DAPT (N-[N-(3,5-difluorophenacetyl-l-alanyl)]-(S)-phenylglycinet-butyl ester), a γ-Secretase inhibitor able to hamper Notch1 activation following ligand binding [40], has been also verified.

MATERIAL AND METHODS

Cells and Culture Conditions

HL-60 leukemic cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 -air using RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics and 10% foetal calf serum (FCS) (Biochrom AG Seromed, Berlin, Germany). Growth rate and cell viability were monitored daily by the trypan blue exclusion test (Sigma-Aldrich S.p.A., Milano, Italy).

Cell Treatments

HNE (Calbiochem, La Jolla, CA, USA) was added to cell suspension (200,000 cell/ml) at the final concentration of 1 μM. The aldehyde was added at regular intervals of time (45 min) up to 10 treatments (the overall time of exposure to the aldehyde was 7.5 hours). No washing steps were performed.

Dimethyl sulfoxide (DMSO, Sigma-Aldrich S.p.A., Milano, Italy), a common inducer of cell differentiation, was added at a concentration of 1.25% to the HL-60 cell suspension (200,000 cells/ml), and used as positive control.

DAPT (N-[N-(3,5-difluorophenacetyl-l-alanyl)]-(S)-phenylglycine t-butyl ester) (Sigma-Aldrich S.p.A., Milano, Italy), the inhibitor of Notch signalling, was added at a concentration of 25 μM, in a solution containing 0.025% DMSO, to the HL-60 cell suspension (200,000 cells/ml).

Butyrate (BUT, Sigma-Aldrich S.p.A., Milano, Italy) 1 mM and the 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich S.p.A., Milano, Italy) 10 ng/ml were added to the HL-60 cell suspension (200,000 cells/ml) to induce apoptosis.

RNA Isolation and Semi-Quantitative RT-PCR Analysis

RNA analyses were performed by a semi-quantitative PCR method as previously described [38]. Briefly, the experimental strategy included the following precautions: (I) the number of PCR cycles was kept low in order to obtain an exponential amplification of PCR products; (II) all results were standardised using the signal obtained with GAPDH (glyceraldehyde 3-phosphate dehydrogenase); (III) all experiments were performed with at least three independent cDNA preparations; (IV) to control for DNA contamination, primers were designed to span at least one exon-intron boundary. Total RNA was isolated using the RNA fast Kit (Molecular System, Genenco, Milano, Italy). cDNA synthesis was performed with 4 μg of total RNA in a reaction volume of 40 μl containing 1.25 μg of random primers, l mM of dATP, dGTP, dCTP and dTTP (Invitrogen, Milano, Italy), 66 units of RNAguard (Amersham Biosciences, Cologno Monzese, Milano, Italy), 8 μl of 5x first strand buffer, 10 mM DTT, 300 units of MMLV reverse transcriptase (Invitrogen, Milano, Italy). Samples were incubated for l h at 37 °C and the reaction was stopped by heating for l0 min at 95°C. PCR reactions were performed in a GeneAmp PCR System 9600 (Perkin Elmer), with 1 μl of cDNA reaction mixture in a volume of 50 μl containing 200 μM of dATP, dTTP, dGTP and dCTP, 1 μM of 5'- and 3 '-primer and 1.25 units of TAQ DNA polymerase (Finnzymes, Milano, Italy). Negative controls contained water instead of cDNA. The

primer pair sequences used for PCR amplification and the numbers of PCR cycles done are indicated as follow:

Notch1 – 25 cycles

(forward primer): 5'- CGCCTTTGTGCTTCTGTTCT-3'

(reverse primer): 5'- CCCACTCATTCTGGTTGTCG-3'

amplifying a 215-bp fragment.

Hes1 – 26 cycles

(forward primer): 5'- ACGACACCGGATAAACCAAA-3'

(reverse primer): 5'- CGGAGGTGCTTCACTGTCAT-3'

amplifying a 200-bp fragment.

CD11b - 33 cycles

(forward primer): 5'-GGGCTCTGCTTCCTGTTTG-3'

(reverse primer): 5'-CTGCGTTATTGGCTTCACC-3'

amplifying a 300-bp fragment.

c-Myc - 26 cycles

(forward primer): 5'- GAGACAACGACGGCGGTG-3'

(reverse primer): 5'-GCTCGTTCCTCCTCTGGC-3'

amplifying a 788-bp fragment.

GAPDH – 25 cycles

(forward primer): 5'-GTCGGAGTCAACGGATTTGG-3'

(reverse primer): 5'-GGGTGGAATCATATTGGAACATG-3'

amplifying a 119-bp fragment.

A l0 µl sample of the PCR reaction mixture was separated on a 1% agarose gel and amplification products were stained with GelStar nucleic acid gel staining (FMC BioProducts, Rockland, ME USA). Densitometric analysis was perfomed by using a software program (Multi-Analyst, version 1.1, BioRad Laboratories, Segrate, Italy).

Preparation of Total Extracts and Western Blot Analysis

Total proteins (60 µg/lane) were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Membranes were probed with the antibodies to Notch1 (sc-6014) and to β-actin (sc-10731) were from Santa Cruz Biotechnologies Inc. Antibodies and signal was detected with the enhanced chemiluminescence (ECL) kit (Amersham Biosciences, Cologno Monzese, Milano, Italy). Signal quantification was performed following image acquisition by means of EDAS 2400 System (Kodak) and subsequent analysis by Kodak 1D Image Analysis System.

Apoptosis

After treatments, cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, 1 mg/ml in methanol) for 30 min at 37 °C in dark. Slides were then washed in PBS, mounted in Mowiol (10% in PBS) and examined under a fluorescence microscope equipped with a UV light filter (Dialux 20, Leitz). Apoptotic cells were defined on the basis of nuclear morphology changes such as chromatin condensation and fragmentation (bead-like formation). Ten different fields were randomly selected for counting 300 cells. The percentage of cells with fragmented nuclei was then calculated.

RESULTS

HNE Affects HL-60 Cells Growth and Differentiation

HL-60 cells were treated with 1 µM HNE for 10 times at regular intervals of time (45 min), according to the protocol previously used to induce HL-60 cell differentiation [41]. This experimental procedure had also been extended to other tumour cell lines, to assess the differentiative ability of HNE, such as SK-N-BE human neuroblastoma cell line [42] and SaOS2 human osteosarcoma cells [43]. 1.25% DMSO was used as positive control of HL-60 cell differentiation.

HNE was able to significatively inhibit HL-60 cell growth since 48h, albeit at a lesser extent with respect to DMSO (Fig. **2A**). Analysis of the differentiation level, assessed by RT-PCR of the surface marker CD11b indicated that the two compounds share the ability to induce differentiation (Fig. **1B**). DMSO induced a higher and long-lasting increase of CD11b, in comparison to HNE.

HNE Negatively Regulates Notch1 Pathway

Notch is involved in hematopoietic progenitor cells differentiation and growth. In order to investigate if the Notch pathway could contribute in mediating the biological effects induced by HNE treatment, first we analyzed changes in Notch1 expression in HNE-treated HL-60 cells.

For this purpose, the RT-PCR analysis of Notch1 mRNA was performed in HL-60 untreated cells or treated with HNE, DMSO at the indicated times (Fig. **2A**). Results clearly show that HNE affected cells ability to transcribe Notch1 at very early experimental times: about 50% reduction in transcription level was evident at 3 and 8h, whereas at 24h Notch1 expression returned to the control level and remained unaltered for the residual experimental times.

In Fig. (**2B**), Western blot analysis of Notch protein is represented. Changes in Notch1 mRNA expression paralleled with the Notch1 transmembrane (TM) and Notch1 intracellular (IC) protein amount, at 3h and 8h after the beginning of HNE treatment (Fig. **2B**); later, consistently with mRNA variations, HNE did not significatively affect Notch1 protein levels.

As far as the effect of DMSO on Notch1 expression, it is noteworthy to observe that, although DMSO was more effective with respect to HNE in inducing differentiation and in inhibiting cell proliferation, it reduced Notch1 mRNA content only at 3h (Fig. **2A**) without affecting the protein level (Fig. **2B**).

The study of the Notch downstream pathway was performed by analyzing the expression of the Notch transcriptional target, HES1. Results indicated that HES1 was negatively regulated by HNE according to changes of Notch1 transcription level, at 3 and 8h (Fig. **2A**). Besides, DMSO

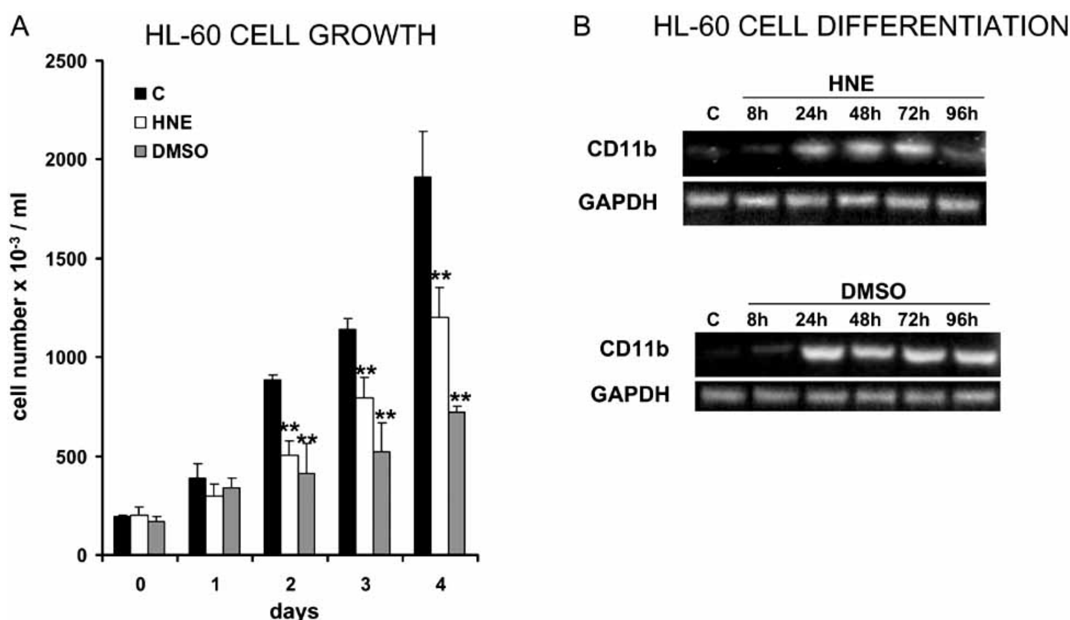

Fig. (1). Panel A: HL-60 cell proliferation after 10 repeated treatments with 1 μM HNE and 1.25% DMSO. Variance analysis: **: p<0.01. **Panel B:** mRNA levels of CD11b (differentiation marker) determined by RT-PCR in HL-60 cells treated with HNE and 1.25% DMSO at the indicated times. GAPDH signal was used to normalize the CD11b signal both in HNE and DMSO treated cells.

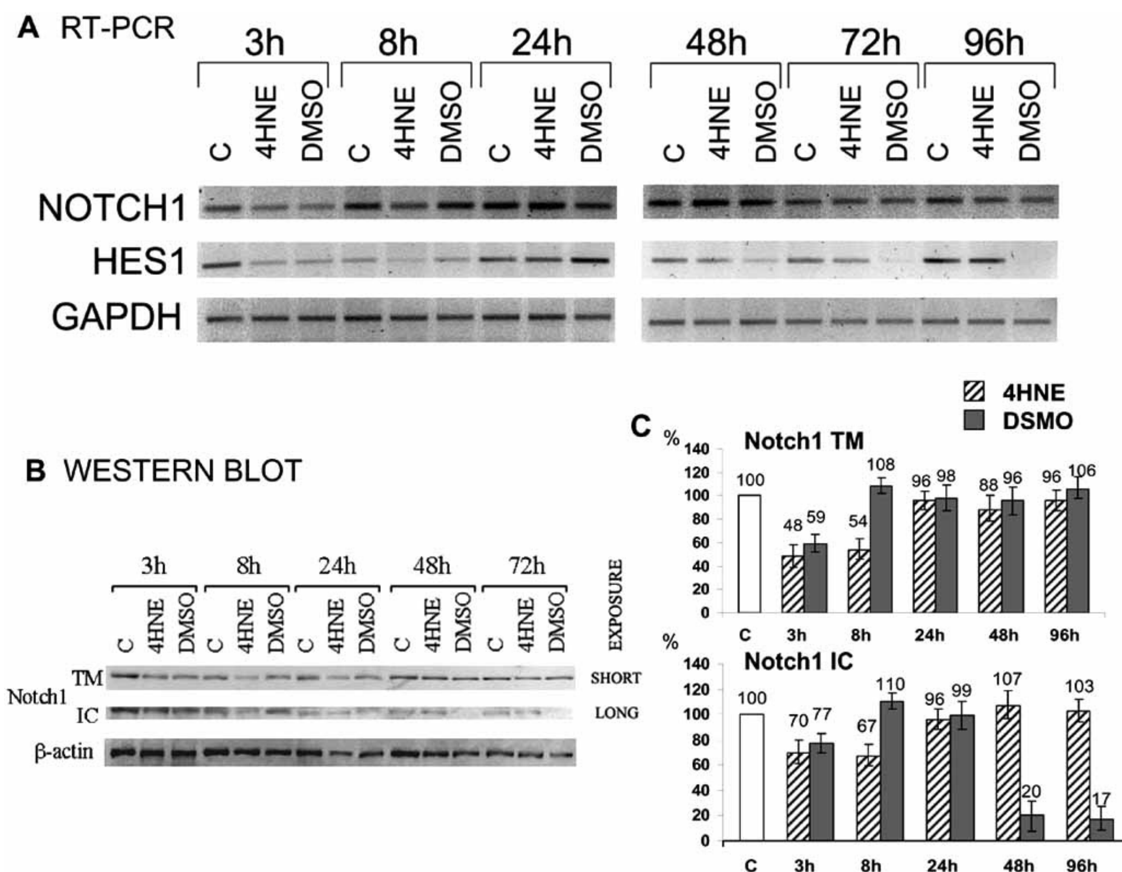

Fig. (2). Panel A: Notch1 and HES1 mRNA levels determined by RT-PCR in HL-60 cells treated with 1 μM HNE 1.25% DMSO at the indicated times. GAPDH signal was used to normalize expression of both genes. **Panel B:** Notch1 protein expression. To allow the detection of both trasmembrane (TM) and intracellular (IC) Notch1 forms, two different exposure times from the same filter are shown (Notch1 TM,120kDa, detectable at low exposure; Notch1 IC, ~105 kDa, detectable at high exposure). **Panel C:** Band intensity of Notch1 IC and TM proteins (western blot in Fig. **2B**), quantified by densitometric scanning using Kodak-1D© system. Data was normalized using the β-actin signal and indicated as the mean ± SEM of three independent experiments.

treatment demonstrated a further later effect on HES1 mRNA expression, which was significantly down-regulated from 48 to 96h. The late changes of HES1 expression after DMSO treatment (Fig. **2A**) were associated to those occurring at the 105 kDa band, corresponding to the Notch1- IC form (Fig. **2B**), clearly down regulated cell growth in HL-60 cells treated with DMSO from 48h until the end of experiment (72h).

Notch1 is Involved in the Inhibition of Cell Growth by HNE

We postulated that, if Notch1 withdrawal is fundamental in cell growth inhibition and triggering of HL-60 cells differentiation, the HNE biological effect could be increased by a contemporary forced Notch1 inhibition. For this purpose the HL-60 cell line was treated with HNE in the presence and in the absence of 25 μM DAPT, a γ-Secretase inhibitor widely used as Notch inhibitor. Results, reported in Fig. (**3A**), indicate that DAPT slightly inhibited cell growth, with a lesser extent compared to HNE alone. Moreover, the two treatments in combination displayed an additive effect suggesting a role for Notch1 in sustaining HL-60 cells growth.

Moreover, to verify if cell death could contribute to the reduction of cell growth, we analyzed the effect of HNE and DAPT on apoptosis (Fig. **3B**). DAPT and HNE alone did not induce apoptosis and a little, but not significant, increase in apoptotic cells was observed when HNE and DAPT were contemporaneously added to the culture medium (from 6,5% to 12,5%). Necrosis also did not occur in any experimental condition tested, as verified with the trypan blu test (data not shown). The combined treatment of butyrate and TPA, used as positive control for apoptosis, induced a significant increase (by 41%) of apoptotic cells. By contrast, the analysis of differentiation induction by examining variations in the expression levels of CD11b marker by RT-PCR (Fig. **3C**), demonstrated that DAPT did not induce differentiation. Moreover, the combined treatment with HNE and DAPT slightly reduced the CD11b expression level, if compared to those of cells treated with HNE alone.

Several reports have recently highlighted the key role of c-Myc as a Notch1 transcriptional target in cell cycle and apoptosis regulation. Down-regulation of c-Myc is also a common effect displayed by HNE in several cell types [34, 37, 44]. Therefore, we analyzed c-Myc expression after

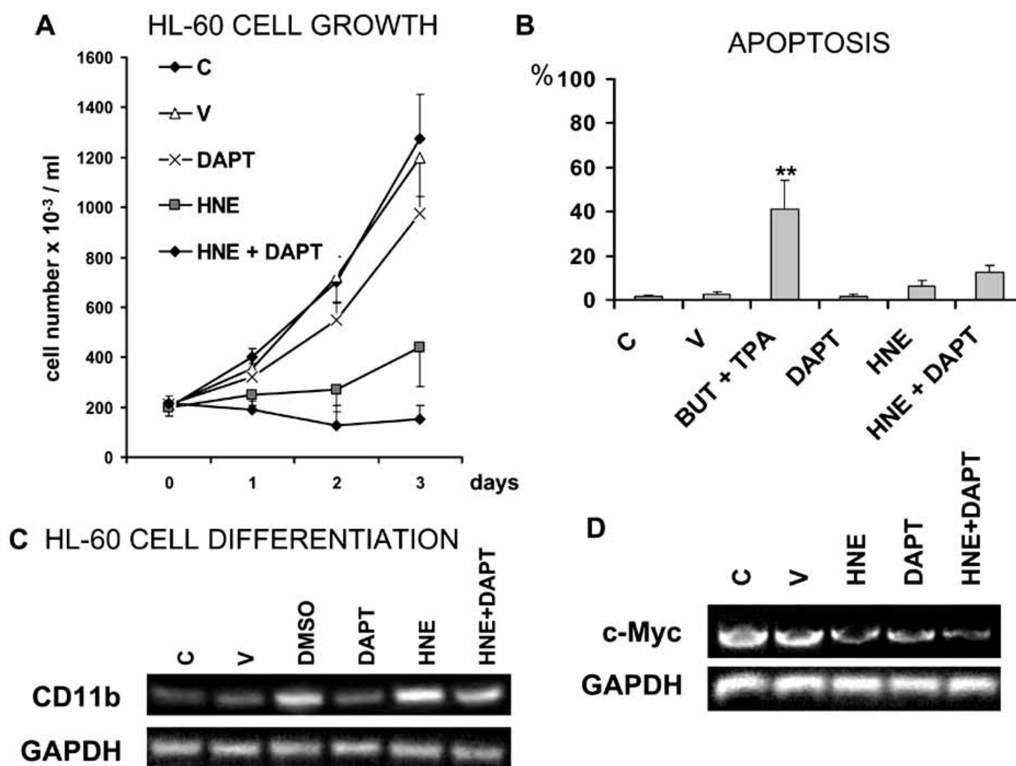

Fig. (3). Panel A: HL-60 cell proliferation of untreated cells (Control, C), cells treated with DMSO 0.025% (vehicle of DATP solution, V), after repeated treatments with 1 μM HNE, 1.25% DMSO, 25 μM DAPT, and contemporary treatment with 1 μM HNE repeated treatments and 25 μM DAPT (HNE+DAPT). **Panel B:** Induction of apoptosis in HL-60 untreated cells (Control, C), cells treated with DMSO 0.025% (vehicle of DATP solution, V), cells treated with Butyrate plus TPA as positive control (BUT+TPA), after repeated treatments with 1 μM HNE, 1.25% DMSO, 25 μM DAPT, and contemporary treatment with 1 μM HNE repeated treatments and 25 μM DAPT (HNE+DAPT). Apoptosis was detected by DAPI staining after 24h. Results are expressed as percentages and indicate mean values ± SEM of three independent experiments for each condition. Statistical analysis: $**p < 0.01$ VS control. **Panel C:** CD11b mRNA levels determined by RT-PCR in HL-60 untreated cells (C), treated with 0.025% DMSO (vehicle of DATP solution, V), 1.25% DMSO, 25 μM DAPT, 1 μM HNE, HNE plus DAPT (HNE+DAPT) at 24h. GAPDH signal was used to normalize gene expression. **Panel D:** c-Myc mRNA levels determined by RT-PCR in HL-60 untreated cells (C), treated with 0.025% DMSO (vehicle of DATP solution, V), 1 μM HNE, 25 μM DAPT, HNE plus DMSO (HNE+DMSO) at 24h. GAPDH signal was used to normalize gene expression.

treatment with HNE, DAPT and the contemporary treatment with both substances (Fig. **3D**).

According to previous data, c-Myc expression was clearly down regulated after HNE treatment (Fig. **3D**). DAPT also was able to inhibit the expression of this oncogene. Moreover, the combined treatment with DAPT and HNE displayed an additive effect in inhibiting c-Myc expression

DISCUSSION

HNE is an intracellular product of lipid peroxidation induced by oxidative stress [29, 31]. Low levels of lipid peroxidation in tumor cells make HNE production often undetectable, suggesting that HNE may be involved in the control of cell proliferation. In fact, HNE treatment of several tumour cell types inhibited cell growth and, occasionally, reactivated the differentiation program [36, 41, 43, 44]. In particular HL-60 cell differentiation induced by HNE has been well characterized in our laboratories [35, 41]. The aim of this work was to explain the contribution of the oncogene Notch1 to the HNE anti-proliferative and pro-differentiative effect in the human myelogenous cell line HL-60.

HNE treatment induced in HL-60 cells the downregulation of Notch1 mRNA and protein at 3h and 8h, whereas at later points (24h-96h) the expression returned to control values. This suggests that Notch1 down-regulation lasted as long as the aldehyde was present in the medium. Consistently with this inhibition, Hes1, a principal effector of the Notch pathway, was also inhibited at the same times (3-8 h) in HNE-treated cells.

DMSO, a widely used differentiation inducer, showed a clearly longer-lasting effect on cell growth inhibition and differentiation induction, with respect to HNE. This could be due to DMSO chemical properties that make it stable for a longer time than HNE.

In DMSO-treated cells, we observed an early and transient inhibition of mRNA Notch1 and Notch1-TM protein. However, since the Notch1-IC protein was down-regulated at later times (48h-72h), when Notch1-TM protein content was similar to control, we suggest that DMSO may post-translationally affect Notch1, possibly by regulating γ-Secretase activity. Hes1 down-regulation at the same times, confirms that DMSO is also able to affect Notch1 signalling. The treatments with both HNE and DMSO, were associated with Notch signalling inhibition in HL-60 cells, suggesting a role of this gene in HL-60 proliferation.

This result is consistent with those obtained by other laboratories in several types of myelogenous leukemia cells after treatment with common differentiation inducers: i.e. HMBA, which affects MEL cells growth and differentiation by downregulating Notch1 [17].

In an attempt to better elucidate the Notch1 role in HNE-induced growth-arrest and differentiation, we verified if the HNE biological effect in HL-60 cells could be enhanced by a contemporary forced Notch1 inhibition, elicited by DAPT. DAPT treatment alone did not affect cell growth but the combination of treatments with HNE and DAPT, resulted in an enhanced inhibition of cell proliferation, more evident than that induced by HNE alone. These effects were not accompanied by cell death (necrosis or apoptosis). DAPT alone was not able to induce differentiation and the contemporary delivering of HNE and DAPT resulted in a reduction of the CD11b increase, determined by HNE alone.

Changes in c-Myc expression determined by HNE and DAPT are in agreement with the reduction of cell growth. In fact, HNE, as well as DAPT, reduces c-Myc expression and display an additive effect if simultaneously delivered. We previously demonstrated that HNE-induced c-Myc inhibition is mediated by the pRb/E2F pathway [39]; now we show evidence that Notch1 down-regulation may also contribute to c-Myc inhibition promoted by HNE, even if further investigations should be perfomed for a direct demonstration of this hypothesis. These data perfectly agree with the observed effect of Notch withdrawal on HL-60 cells proliferation; in fact it has been recently reported that c-Myc is a direct Notch1 transcriptional target and is mainly responsible for Notch1 influence on cell proliferation [45-47]. Similarly, another Notch1 target gene, related to cell cycle regulation, Cyclin D1, is downregulated after HNE treatment [38] further supporting the anti-proliferative effect of Notch signalling inhibition during HNE treatment.

The role of Notch signalling in AML is still under debate and some evidence suggest the complexity of Notch relation with the local molecular and biological environment. A study from Tohda and collaborators [48] demonstrated that the activation of Notch signalling in primary acute myeloblastic leukaemia cells tends to induce differentiation, but several other literature data sources are in contrast with these results. In fact, Notch1 signalling activation in myelogenous leukemia obtained by delivering Notch ligands or the constitutively active Notch1 form, is associated with delay or suppression of cell differentiation, induced by pro-differentiative compounds [16, 18, 49]. In particular for HL-60 cells, Carlesso and collaborators [50] reported that the treatment with differentiation inducers, such as ATRA or TPA, in those cells over-expressing a constitutively active form of Notch1, induce a delayed acquisition of the differentiation marker CD11b, despite the preserved proliferation rate. Results obtained by inhibiting Notch signalling are not homogeneous; down-regulation of Notch caused MEL cells to abort the differentiation program and undergo apoptosis [17], on the other hand expression of antisense Notch-1 induced spontaneous erythroid maturation in K562 [18].

Our work indicates that Notch signalling inhibition occurring during HNE treatment has a part in HNE inhibition of HL-60 cell growth; by contrast Notch withdrawal does not affect HL-60 apoptosis and reversed HNE-induced cell differentiation. Our results, partially in agreement with literature data, underline the complexity of the Notch role in regulating biological processes.

The Notch signalling role is specific not only in relation to the cell type, but different pro-differentiative inducers have an apparently opposite effect on Notch signalling in the same cell type. Therefore, we suggest that the outcome of Notch transcriptional activity could be determined by the specific gene expression profile elicited by different agents. Further investigations should be performed for a better comprehension of this point.

In conclusion, targeting Notch signalling is becoming a promising approach in cancer therapy; γ-Secretase inhibitors are under study, alone or in combination with more common chemotherapeutic drugs [51, 52]. This paper adds new information in this field, confirming the importance of Notch1 signalling and the necessity to precisely understand the result of its interaction with antitumor agents in AML.

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