The pro-apoptotic factor Nix gene is co-expressed with Bcl-xL during terminal erythroid differentiation.

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ABSTRACT
Transcriptional profiles of cultured primary human erythroid cells were examined to identify those genes involved in the control of erythroid growth during the terminal phase of maturation. Our in silico screening strategy indicated that a hypoxia-inducible, pro-apoptotic member of the Bcl-2 gene family called Nix is expressed during erythropoiesis. We next performed Northern blot analyses and determined that the 1.4 kb Nix transcript is expressed at relatively low levels among erythroleukemia cells compared with high levels detected in reticulocytes. PCR-based transcriptional patterning confirmed the increased expression of Nix during human erythropoiesis with a pattern similar to Bcl-xL and glycophorin A and opposite that of Bcl-2. Western analyses revealed lower than expected Nix protein levels among the more differentiated cells that were associated with increased proteosomal degradation of Nix compared with Bcl-xL. The expression of both Nix and Bcl-xL proteins decreased relative to the GAPDH control upon removal of erythropoietin (EPO) from the culture medium. Immunocytochemical analyses demonstrated a similar perinuclear mitochondrial expression pattern for both proteins in hemoglobinized precursors. On the basis of these data, we propose that the pro-apoptotic factor Nix is a highly regulated effector of growth during terminal erythroid maturation. (e-mail: jm7f@nih.gov.)
INTRODUCTION

Human erythropoiesis encompasses differentiation of hematopoietic stem cells into recognizable erythroid blasts, culminating with enucleation to form the reticulocytes. Effective terminal maturation depends upon intracellular signaling networks that regulate cell growth and apoptosis. In particular, regulated expression of the anti-apoptotic gene Bcl-xL is thought to mediate erythroblast viability during terminal maturation (1,2). Significantly increased expression of Bcl-xL is also associated with polycythemia (3,4).

To identify additional growth-related genes with regulated expression during terminal erythroid differentiation, we compared the transcriptional profiles of primary human erythroid cells sorted at defined stages of differentiation. We identified several expressed sequence tags (ESTs) with homology to a gene called Nix (Genbank GI: 4138825). Expression of this gene has not been reported previously among erythroid cells. Nix, also known as BNIP3L and BNIP3α, is a pro-apoptotic member of the Bcl-2 gene family located on chromosome 8p21 (5). Nix binds to anti-apoptotic members of the gene family, including Bcl-xL, and has been shown to directly target mitochondrial membranes to induce apoptosis-associated changes (6,7). Since Nix encoded ESTs were identified more frequently among transcriptional profiles from mature erythroid cells compared with those from immature cells, we experimentally tested the informatic-based hypothesis that expression of this pro-apoptotic gene is regulated during terminal erythroid differentiation. For this purpose, we compared expression patterns for Nix and Bcl-xL during adult erythropoiesis. Interestingly, both genes demonstrated a highly regulated, erythropoietin dependent expression pattern during terminal erythroid maturation.

METHODS

Erythroid primary cell culture. Peripheral blood CD34+ cells were isolated from normal donors and cultured in the presence of EPO for differentiation of the erythroid lineage as discussed elsewhere (8).
**Northern Blot Analysis.** A membrane was prepared with 25 µg of total RNA isolated from human reticulocytes, white blood cells, K562 erythroleukemia cells, HEL erythroleukemia cells, and Jurkat cells using Trizol™ (Life Technologies, Gaithersburg, MD) along with 2.5 µg of bone marrow mRNA purchased from BD Biosciences Clontech (Clontech, Palo Alto, CA). An 850 bp probe corresponding to position 169 to position 1019 of Nix mRNA was generated by polymerase chain reaction using NorthernMax Kit (Ambion, Austin, Texas) with the forward primer: 5’ GCCGGCCTCAACAGTTC 3’ and reverse primer: 5’ TGGCATTTGCGGAAAAGA 3’.

**Expression data using RT-PCR.** Total RNA was prepared from 5-10 x 10⁵ cells using Trizol™ reagent (Life Technologies, Gaithersburg, MD), and cDNA was synthesized from 1 µg of total RNA per sample using SuperScript II Kit (Gibco/BRL, Carlsbad, CA) with oligo dT primers according to the manufacturer’s instructions. Expression was then assessed using primers for Nix (forward 5’ CGCCCTGCACAACAAC 3’ and reverse 5’ TCATTGCCATTGCTGCTG 3’), GAPDH (forward 5’ GGCGCCTGGTCA 3’ and reverse 5’ CAAGCTTCCGTTCAG 3’), GPA (forward 5’ CGCACAAACGGGACACATA 3’ and reverse 5’ CAGAGAAATGATGGGCAAGTT 3’), Bcl-2 (forward 5’ GTGTGGAGAGCGTCAACC 3’ and reverse 5’ GCTGGGGCCGTACAGTT 3’). Bcl-xL was amplified with primers specific for the longer spliced form (forward 5’ GGATGGCCACTTACCTGA 3’ and reverse 5’ CGGTTGAAGCGTTCCTG 3’). All PCR primers were designed to span an intron, and all products were amplified using two-step 94°C/68°C conditions.

Quantitative real-time RT-PCR was performed using an ABI Prism 7700 sequence detection system (PE Applied Biosystems; Foster City, CA). Amplification of specific PCR products was performed in a total reaction volume of 50 µL containing 10-µL (100ng) cDNA template, forward and reverse primers, dual-labeled fluorogenic internal probe and 1x TaqManR Universal PCR Master Mix reagents. Dual-fluorescent nonextendable probes labeled with 6-
carboxyfluorescein (FAM) at the 5’ end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3’ end were used for detection. The primers and probes for Nix (forward 5’ AAAATGAGCAGTCTCTGCCCC 3’ and reverse 5’ TGCTGCTGTTGATGGGACGT 3’, probe 5’ 6FAM-CGGCCTCAACAGTCTGGGTG-TAMRA 3’) and Bcl-xL (forward 5’ GAATGACCACCTAGAGCCTTGG 3’ and reverse 5’ TGTTCCCATAGAGTTCCACAAAAG 3’, probe 5’ 6FAM-TCCAGGAGAAGGCGGCTGG-TAMRA 3’)

were designed using the PRIMER EXPRESS software (PerkinElmer Life Sciences, Boston, MA). Amplifications were performed at 95°C 10 minutes and amplified for 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Serial dilutions of cDNA were amplified in each experiment for calculation of the relative mRNA expression levels of the target genes according to the manufacturer’s protocol.

Western Blot Analysis. Harvested cells were lysed in a buffer containing 0.15 M NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton (v/v) X-100, 0.05 M Tris-HCl, and protease inhibitors, and the protein extracts (30 µg /well) were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. Nix was detected with rabbit anti-human Bnip3L (Nix) polyclonal antibody (1:500; Exalpha Biologicals, Boston, MA) followed by an anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, #sc-2004). Mouse monoclonal anti-Bcl-xL and anti-Bcl-2 antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, #sc-8392 and #sc-7382) were used at a 1:200 dilution and detected with anti-mouse IgG-HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, #sc-2005). The membranes were developed with an enhanced chemiluminescence reagent according to the manufacturer’s instructions (Amersham Life Science, Little Chalfont, UK). For protein stability analyses, the translation inhibitor cycloheximide (CHX, 200 µM final concentration; Sigma, St. Louis, MO) versus the protease inhibitor lactacystin (LAC, 5 µM final
concentration; Calbiochem, La Jolla, CA) were as added for 48 hours beginning on culture day 12 prior to Western analyses.

**Immunocytochemical Staining.** Cytospin preparations were made with primary human erythroid precursors cells obtained after two weeks in culture. The cell slides were fixed in chilled Acetone for 10 minutes, air dried, and subsequently incubated for 5 minutes in 1% hydrogen peroxide to quench any endogenous peroxidase activity. After blocking in 2% BSA-PBS for 1 hour at room temperature, the slides were incubated with rabbit antihuman Nix (Bnip3L) antibody (Exalpha Biologicals, Boson, MA) at a 1:100 dilution versus mouse antihuman Bcl-xL antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:50 dilution (overnight at room temperature). After washing, they were immunostained using an automated avidin-biotin HRP detection system (Ventana Medical Systems, Inc., Tucson, AZ).

**Measurement of apoptosis by flow cytometry.** After washing twice with PBS, 1 x 10⁶ cells were resuspended in 0.5 mL of binding buffer (10 mmol/L hepes/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂). Fluorescently labeled Annexin V (FITC-AnV) was added at a final concentration of 0.5 µg/mL, and the cells were incubated 10 minutes at room temperature according to the manufacturer’s protocol (Immunotech, Marseille, France). Flow cytometric analyses were performed using an EPICS ELITE ESP flow cytometer (Beckman Coulter, Hialeah, FL). In each experiment, at least 10,000 cells were analyzed using argon laser excitation and bandpass emission filters: 525 nm for fluorescein (FITC).

**RESULTS**

**Erythroid expression of Nix mRNA.** We initially sequenced several Nix encoded cDNA clones from erythroid cells, and identified a 1366 bp transcript (Genbank ID: AF452712) with an additional 15bp at the 5' terminus resulting in a longer first exon (197 bp) than Nix (Genbank ID: AF067396). The additional 15bp
in the 5'UTR region indicates transcription slightly upstream of that reported previously, but the predicted coding regions and exon splicing patterns are not changed. Of note, a transcript containing a much longer 3'UTR has also been reported (Genbank ID: AL132665; 3481 bp). Northern blot analysis revealed two bands with variable intensities at around 4.0 kb and 1.4 kb (Figure 1). This dual banding pattern is present in most tissues, and slight variations in the molecular weights of these bands have been identified elsewhere (7,9). The larger transcript is usually expressed at equivalent or higher levels (10), and this pattern was demonstrated in the leukemia cell lines and peripheral blood leukocytes (Figure 1). The lower molecular weight band was significantly more intense in bone marrow and reticulocytes. The reversal of the intensities of the two bands, especially among reticulocytes suggests increased expression of the 1.4 kb Nix transcript during erythroid maturation. The significance of the preferential expression of the 1.4 kb Nix transcript in reticulocytes is currently unknown.

**Nix transcription during terminal erythroid differentiation.** As stated above, ESTs encoding this gene were more abundant among erythroid precursor versus progenitor cell populations (1.0% of precursor EST versus 0.02% progenitor EST). Hence, we next sought to compare the pattern of Nix expression with other genes having regulated expression during terminal erythroid differentiation. For this purpose, primary human CD34+ cells were cultured in the presence of EPO over a two-week period (8). We have previously demonstrated that this culture model results in rapid growth of erythroid progenitors during the first week followed by reduced proliferation and terminal differentiation during the second week (8). RT-PCR was performed every other culture day to examine gene activity during differentiation. This experimental approach provides a pattern of transcriptional activity for individual genes during the differentiation process, and a qualitative comparison of separate genes amplified from the same templates. Those patterns reflect relative gene activity within cellular populations rather than individual cells. As shown in Figure 2A, the transcription of the GAPDH housekeeping gene remained relatively stable throughout the differentiation
process. The other genes demonstrated graded patterns in their signal intensities suggesting erythroid-regulated expression. The expression of Nix was relatively low during the early stages of differentiation, with a subsequent increase in expression as the cells matured. This pattern is consistent with the Northern blot in Figure 1 in demonstrating a relatively higher level of Nix transcription among more mature cells. The expression patterns for the EPO-dependent Bcl-xL and Glycophorin A (GPA) genes were also tested and demonstrated similar differentiation-related increases. In contrast, the expression of Bcl-2 sharply declined as the cells matured. Erythroid expression of Bcl-2 has also been shown elsewhere to decrease in response to EPO (11). Quantitative PCR was also performed to confirm the transcriptional expression patterns of Nix and Bcl-xL (Figure 2B). The overall expression patterns of Nix and Bcl-xL similarly increased with peak expression on day 12 relative to GAPDH. After day zero, Nix mRNA levels were slightly greater than Bcl-xL throughout the culture period.

**Nix and Bcl-xL protein expression, stability and localization.** Based upon the transcriptional patterns of Nix and Bcl-xL during erythroid maturation, we next compared their protein expression patterns (Figure 3). Two bands were hybridized by the Nix polyclonal antibody with molecular weights of approximately 38 and 40 kDa respectively. The lower molecular weight Nix signal demonstrated a higher intensity in all samples. Both bands increased rapidly from days 2 to 6 followed by a gradual decrease in signal intensity during the second week in culture. This pattern corresponds to peak Nix protein expression among proerythroblasts at the transition to lower growth rates and terminal differentiation (8). This Nix expression pattern was different from transcriptional pattern demonstrated above (Figure 2) as well as the protein expression pattern of Bcl-xL (Figure 3; middle panel). Unlike Nix, the protein expression of Bcl-xL protein correlated closely with its transcriptional pattern. In the case of Bcl-xL, the signal gradually increased until late in the culture period. The Bcl-2 protein expression pattern was consistent with the mRNA expression pattern with barely detectable levels during the second week at the terminal phase of maturation. On
day 14, the signal intensities for Nix, Bcl-xL, and the GAPDH control were all decreased compared with day 12.

To further study the decreased Nix protein expression late in the culture period compared with increased mRNA levels during the same period, we inhibited protein translation and proteosominal degradation on days 12-14. As shown in Figure 4, the levels of Nix and Bcl-xL proteins were both decreased on day 14 compared with day 12 in control samples. Bcl-xL levels were unchanged in the presence of the proteosominal inhibitor lactacystin, but the protein translation inhibitor cycloheximide caused a significant reduction in Bcl-xL. This pattern suggests Bcl-xL protein levels are regulated primarily at the level of translation rather than proteosominal degradation. In contrast to Bcl-xL, Nix levels increased in lactacystin and in cycloheximide. The rise in Nix levels in the setting of inhibited proteosominal degradation as well as the seemingly paradoxical rise in Nix associated with translational inhibition has been described elsewhere in proteins with expression levels regulated primarily through proteolysis (12,13). Cycloheximide is thought to increase the stability and cellular levels of some proteins by inhibiting their degradation. These data suggest that the differences in Nix mRNA and protein levels during terminal erythroid differentiation arise primarily from increased proteosominal degradation in those cells.

Immunocytochemistry analyses were also used to demonstrate the localization of Nix and Bcl-xL during terminal erythroid differentiation. Mitochondrial localization of these two proteins has been reported in other cells (14). Cytospin preparations of erythroid precursor cells were stained and compared for this purpose (Figure 5). Giemsa staining confirmed erythroid maturation with hemoglobinization, and no peroxidase staining (rust color) was detected in the absence of a primary antibody (Figure 5A, 5B). Hybridization with either Bcl-xL (Figure 5C) or Nix (Figure 5D) primary antibodies resulted in a nearly identical “ringed” perinuclear pattern in each cell. Examination of over one hundred cells revealed a pancellular distribution (detected in >90% of the cells) of Nix and Bcl-xL. Mitochondrial-specific dyes demonstrated a similar perinuclear
pattern in these cells (not shown). This perinuclear pattern is highly reminiscent of abnormal iron accumulation in the mitochondria of ringed sideroblasts.

**EPO-dependent expression of Nix and Bcl-xL.** It has been demonstrated elsewhere that expression of Bcl-xL is dependent on EPO signaling during the erythroid differentiation (15,16). We therefore addressed in our model system whether EPO signaling also affected the regulation of Nix expression during terminal erythroid differentiation (Figure 6). CD34+ cell populations grown in EPO during the first week were transferred to culture medium lacking supplemental EPO during the second culture week (days 7-14). After 48 hours in the absence of EPO (Figure 5, Day 9), Nix expression was detected at nearly the same level as the EPO-containing control. However, the Nix signal decreased to lower levels in the absence of EPO by day 14. Compared with Nix, the response in Bcl-xL expression to EPO withdrawal was profound with a clearly decreased signal on day 9 and no detectable signal by day 14. While the levels of both proteins were reduced in response to EPO withdrawal, these data suggest that the balance of Nix: Bcl-xL proteins increased to Nix dominant levels in association with Epo withdrawal. Annexin V based flow cytometry was used to correlate the changes in Nix and Bcl-xL levels with levels of apoptosis. On day 9, apoptosis was detected with 8.2% of the EPO (-) cultures compared to 6.1% of the cells maintained in EPO. Differentiation of the cells resulted in a small increase in Annexin V staining by day 14 even in EPO containing medium, but the proportion of cells exhibiting high levels of Annexin V signal remained low at 10.2%. By comparison, Epo withdrawal on day 7 resulted in apoptosis of over 80% of the cells by day 14 as evidenced by high levels of Annexin V staining.

**DISCUSSION**

This study was initiated from our recognition of increased representation of Nix mRNA within transcriptional profiles of maturing erythroblasts. Although constitutive Nix gene expression is well recognized (5,7), developmental or differentiation-related expression of this gene has not been reported previously.
In addition, Nix expression within primary hematopoietic cells including the erythroid lineage has not been reported. The Nix gene is well conserved among multicellular organisms, and a Nix orthologue is reportedly involved in apoptosis in *C. elegans* through interaction with CED-9 and CED-3 (17). In mammals, Nix belongs to a subset of pro-apoptotic mitochondrial proteins within the Bcl-2 protein family that interact with the pro-survival members Bcl-2 and Bcl-xL (6, 9, 18). Interestingly, Nix overexpression reportedly induces apoptosis in a delayed fashion compared with most other pro-apoptotic members of the Bcl-2 family (7). Structurally, Nix contains a PEST domain, a putative BH3 domain, and a C terminal TM domain. *In vivo*, Nix homodimerizes or associates with other Bcl-2 members to form heterodimers (6). Consistent with the data presented here, Nix is usually localized to the mitochondrial membrane, but cytoplasmic localization has been reported elsewhere in association with a splicing variant or loss of the TM domain (19).

Nix gene and protein expression in adult human erythroid cells appears to be highly regulated. Nix mRNA was identified as 1.4 kb and 4.0 kb transcripts here and elsewhere (7). The 1.4 kb transcript was increase relative to the 4.0 kb band only in bone marrow and reticulocyte samples suggesting possible erythroid upregulation. In addition to the strong signal in reticulocytes, the relative increase in Nix signals during erythroid maturation (Figure 2) suggests developmental up-regulation of Nix transcription during terminal erythroid maturation. The increase in Nix with maturation mirrored that of Bcl-xL suggesting transcriptional coregulation of the two genes. In addition to transcriptional control of the gene, the Western blot analyses (Figure 3) suggest post-transcriptional regulation of Nix protein due to higher levels of proteosomal degradation compared with Bcl-xL. This apparent post-transcriptional regulation of Nix protein expression may be mediated through its PEST domain (9, 20). The period having maximum Nix protein in the cells correlates well with the growth transition from rapid proliferation toward terminal differentiation from culture days 6-9 (8). As demonstrated here experimentally, this period also correlates with the transition from Bcl-2 to Bcl-xL expression. This transition may reflect regulated
gene transcription among the surviving erythroid precursors as well as the loss of non-erythroid, Bcl-2 expressing cells during the first week under these culture conditions.

While not studied here, Nix has additionally been shown to be induced by hypoxia in cell lines (10,21). Our informatic examination of the Nix gene promoter region revealed three putative hypoxia response elements (HREs) within the 5' promoter region at -53 bp, -311 bp and -398 bp upstream of the 1.4 kb erythroid transcript (data not shown). HREs bind hypoxia-inducible factor (HIF-1), a transcription factor which functions as a global regulator of oxygen homeostasis and has been shown to play a role in hypoxic control of growth and apoptosis (22). Other erythroid genes, including the transferrin receptor (23) and 5-aminolevulinate synthase (24) are also upregulated by HIF 1 in response to hypoxia. Fas-mediated signals may also affect Nix expression as this pro-apoptotic pathway is involved in terminal erythroid differentiation (25). Hence, Nix expression patterns likely reflect the convergence of several pathways involved in the transcription, translation and stability of gene products in erythroid cells as they undergo terminal maturation.

Bcl-xL expression during erythropoiesis clearly increases and depends upon GATA-1 and EPO signaling during the red cell maturation (1, 2, 15, 16). Our data suggests that the transcription of Nix increases with a pattern similar to that of Bcl-xL and another EPO-dependent protein glycophorin A. In contrast to the similarities of their transcriptional patterns, the levels of Nix and Bcl-xL proteins were less synchronous during terminal erythroid maturation. Nix protein levels increased earlier than Bcl-xL then decreased despite relatively increased levels of mRNA. Upon withdrawal of EPO and subsequent apoptosis, the levels of both proteins were reduced. However, the reduction in Bcl-xL was greater suggesting an increase in the Nix:Bcl-xL ratio in this pro-apoptotic setting. A similarly increased ratio of Nix expression relative to Bcl-xL has been shown previously to correlate with a loss of cell survival in cell lines (7). Whether additional pro-apoptotic mechanisms are induced upon EPO withdrawal is not known. We propose that coordinated expression of pro- and anti-apoptotic
factors is important for the precise regulation of proliferation in steady states of terminal differentiation to prevent either uncontrolled growth or apoptosis. Normal and disease-related imbalances of these factors must then be considered in the settings of ineffective erythropoiesis, stress erythropoiesis, polycythemia, and leukemia.

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**Figure 1. Northern blot analysis of Nix expression.** Membranes were prepared using RNA from bone marrow (lane 1), leukocytes (lane 2), reticulocytes (lane 3), K562 cells (lane 4), HeLa cells (lane 5), and Jurkat cells (lane 6). RNA was hybridized with an 850 bp segment of Nix mRNA. Arrows indicate the molecular sizes of two bands.
Figure 2. Expression of Nix RNA during terminal erythroid differentiation.

CD34+ cells harvested from the peripheral blood of normal donors were cultured in the presence of EPO to induce terminal erythroid differentiation (see methods) and harvested on day 0, 2, 4, 6, 8, 10, 12 and 14 before RNA isolation and RT-PCR. (A) Equivalent amounts of cDNA were amplified with primers specific for
the genes shown on the left. All PCR assays consisted of 30 cycles except GAPDH, which was amplified for 27 cycles, and Bcl-2, which was amplified for 33 cycles, due to low signals. (B) Expression patterns of Nix (black bars) and Bcl-xL (white bars) were confirmed using real-time quantitative RT-PCR (see methods). The relative expression levels (+/- SD) shown are normalized to the levels of GAPDH mRNA detected on the same day.
Figure 3. Nix, Bcl-xL, and Bcl-2 protein expression pattern during terminal erythroid differentiation. Total cell lysates from erythroid primary CD34+ cells on the indicated culture days were subjected to Western blotting analysis. The blots were hybridized with antibodies to Nix, Bcl-xL, Bcl-2, and the GAPDH control respectively.
Figure 4. Comparison of Nix and Bcl-xL protein catabolism. After 12 days in culture 50 µM/mL lactacystin (Lac) versus 200µM/mL cycloheximide (CHX) was added to inhibit protein translation and proteosomal degradation respectively. Whole cell extracts were prepared from cells on culture day 14 and compared with control samples collected on days 12 and 14. Western blot analyses for Nix and Bcl-xL are shown for comparison.
Figure 5. Expression of Nix and Bcl-xL protein in the erythroid precursors cells. Immunostaining was performed on cytospin preparations of primary human erythroid precursor cells (see methods). (A) May-Grunwald-Giemsa staining of air-dried (unfixed) cytospin preparations to demonstrated erythroid maturity. (B) Cells fixed and stained in the absence of a primary antibody (negative control). (C) Cells immunostained with anti-Bcl-xL antibody. (D) Cells immunostained with anti-Nix antibody. Peroxidase (rust color) tagged secondary antibodies were used for detection in panels B-D. (Bar equals 10 microns).
**Figure 6. EPO-dependent expression of Nix and Bcl-xL.** Cells were cultured in EPO on days 0-7, then grown in the presence (+) or absence (-) of EPO from days 7-14. (A) On days 9 and 14, Western analyses were performed on total cell lysates probed for Nix (top), Bcl-xL (middle), or GAPDH (lower) expression. K562 cell lysates (far right) are included for comparison. (B) Matching flow cytometric analyses of Annexin V staining of the cells. The percentage of apoptotic cells (brightly stained with Annexin V) is provided in the upper right corner of each panel.
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