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Overexpression of Id3 induces apoptosis of A549 human lung adenocarcinoma cells

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Abstract

Objectives: Inhibitor of differentiation 3 (Id3) protein has been implicated in the control of multiple cell death signalling pathways and in aetiology of numerous diseases. The aims of this study were to construct a recombinant eukaryotic expression vector (pEGFP/Id3), containing human Id3 (hId3) fused with enhanced green fluorescent protein (EGFP), and to determine effects of ectopic Id3 overexpression, on human lung adenocarcinoma cell (A549) proliferation.

Materials and methods: Human Id3 cDNA was inserted into pEGFP-N1 vector to yield the recombinant eukaryotic expression vector pEGFP/Id3. Cells were transfected with pEGFP or pEGFP/Id3, and proliferation of EGFP-expressing cells was monitored by flow cytometry (FCM) and confocal fluorescence microscopy. RT-PCR, immunoblotting and immunocytochemistry were used to assess Id3 mRNA transcription and protein expression. Apoptosis was evaluated by Annexin V/7-AAD staining and FCM, while nuclear morphology of apoptotic cells was examined using Hoechst 33258 staining.

Results: Over 4 days transfection with pEGFP, the proportion of EGFP-positive A549 cells peaked at approximately 60% by 48 h and remained stable over the next 48 h. In contrast, the proportion of EGFP-positive cells in cultures transfected with pEGFP/Id3 decreased from a peak of 60% at 48 h to <5% at 96 h, suggesting that Id3 expression inhibited cell proliferation or survival. Annexin V/7-AAD and Hoechst 33258 staining revealed significantly higher rates of apoptosis in pEGFP/Id3-transfected cells.

Conclusion: Overexpression of Id3 triggered apoptosis in A549 human lung adenocarcinoma cells, implicating Id3 in negative control of tumour growth. These Id3-induced pro-apoptotic signalling pathways require further study, but this preliminary investigation suggests that Id3 regulation could be exploited in anti-tumour therapies.

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Introduction

Inhibitor of differentiation (Id) proteins belong to the superfamily of helix-loop-helix (HLH) DNA-binding proteins, but lack the basic DNA binding domain and instead function as inhibitors of basic HLH transcription factors. The four members of the Id family in vertebrates (Id1–4) are crucial for coordinated regulation of cell proliferation, differentiation, apoptosis, tumourigenesis and carcinogenesis (1–3). Regulation of Id expression and protein function is under complex regulatory control, and expression profiles differ markedly between cell types and developmental stages (4–7).

Id3 gene was first identified as a serum-inducible immediate early gene in an established murine fibroblast cell line (8). Functional analyses implicated human *Id3* (hId3) in many diverse developmental, physiological and pathophysiological processes, including T- and B-cell development (9–11), skeletal muscle differentiation, and vascular smooth muscle cell proliferation (12–14). The Id3 isoform has biological functions distinct from other Ids; perturbation of Id3 expression has been correlated with a variety of disease states, including cancer (4,6), atherosclerosis (15,16) and autoimmunity (17). In B lymphocyte progenitors, Id3-induced cell growth arrest and caspase-2 dependent apoptosis (9,18). In immortalized human HPV16 E6/7 keratinocytes (KCs), Id3 facilitated caspase-3/9 dependent apoptosis and UVB sensitization (19,20). These results indicate that Id3 acts upstream of

other cell death regulators to induce apoptosis. Furthermore, they highlight Id3 as a possible target for therapeutic intervention in cancer treatment; however, the aforementioned studies were not performed on human tumour cells. As Id3 displays a more complex pattern of expression in tumour cells, we have examined apoptosis in cancer cells overexpressing Id3.

Our preliminary report (21) demonstrated that Id2 and Id3 mRNAs were differentially expressed in several normal and malignant cell lines. Expression of Id3 mRNA was highly variable in different tumour cell types therefore it is unclear whether Id3 is an important regulator of proliferation and differentiation in malignant cells. We have constructed a eukaryotic expression vector encoding a fusion protein of enhanced green fluorescent protein (EGFP) and Id3 (vector pEGFP/Id3), and we have examined the impact of ectopic Id3 overexpression on A549 cell proliferation and apoptosis. Overexpression of Id3 inhibited proliferation and induced apoptosis in this human lung cancer cell line.

Materials and methods

Cell culture

Human lung adenocarcinoma cell line A549 and the human prostate cancer cell line PC-3M were purchased from the American Type Culture Collection. Both cell types were maintained in minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (Hyclone, Logan, UT, USA), 2.0 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were passaged once every 3 days after trypsinization.

Amplification and cloning of human Id3 cDNA

A cDNA fragment coding the full open reading frame of hId3 was amplified from PC-3M cells by RT-PCR. Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. Primers were synthesized based on the published hId3 cDNA sequence (forward: 5′-ATGAAG GCGCTG AGCCCG GTGC-3′, reverse: 5′-ACGGCC GAGTCA GTGGCA AAAGC-3′). Thermocycle settings for PCR amplification included one pre-denaturation step at 94 °C for 5 min, 35 amplification cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s and extension at 70 °C for 50 s, followed by final extension at 72 °C for 7 min. Amplification products were detected by performing 1.5% agarose gel electrophoresis. Resulting fragments were recovered, purified and ligated into the pEGM-T Easy vector (Promega, Madison, WI, USA) to form the pEGM-T/Id3 cloning vector. Proper insertion of Id3 was then confirmed by restriction enzyme digestion and direct sequence analysis (Sangon Engineering Company, Shanghai, China).

Construction of recombinant pEGFP/Id3 plasmid

The hId3 cDNA fragment from pEGM-T/Id3 vector was then subcloned into pEGFP-N1 expression vector (Clontech Laboratories, Inc. Mountain View, CA, USA) to form the recombinant eukaryotic expression vector pEGFP/Id3. A Kozak sequence was introduced before the initiation site (ATG) in the upstream primer and the stop codon was deleted from the downstream primer used for PCR amplification of Id3 cDNA. These Id3 primers (forward: 5′-GGAAT TCGCC ACCAT GAAGG CGCTG AGCCC GGT-3′, reverse: 5′-CGGGA TCCCC GTGGC AAAAG CTCCT TTTGT-3′) were also synthesized with the restriction sites for *Eco*R I and *Bam*H I. *Id3* gene was amplified by PCR using pEGM-T/Id3 as template. Insertion of Id3 cDNA in the pEGFP vector was confirmed by appearance of an ≈400 bp Id3 cDNA fragment, on agarose gels following digestion with EcoR I and BamH I (Promega). Correct Id3 orientation and encoding of EGFP/Id3 fusion protein were further validated by direct DNA sequencing.

Transfection

Transient transfections were performed with lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 24 h before transfection, A549 cells were plated at $0.5–2 \times 10^5$ density, in 24-well plates. This density range was chosen so that cells would reach 90–95% confluence by initiation of transfection. pEGFP/Id3 plasmids and Lipofectamine 2000 were each diluted separately in 50 μl of serum-free Opti-MEM (Gibco BRL, Invitrogen, Carlsbad, CA, USA) and incubated for 5 min at room temperature. The two solutions were mixed at 0.8 μg plasmid to 2 μl Lipofectamine 2000 and incubated for 20 min at room temperature. A total of 100 μl of this mixture was added to 400 μl of culture medium in each well. Cells were incubated at 37 °C in a 5% CO_2 incubator for 18–96 h, depending on the specific experiment, and then tested for transgene expression.

Analysis of EGFP and EGFP-Id3 fusion protein expression

RT-PCR. Forty-eight hours after transfection, cells were harvested and washed three times in phosphate-buffered saline (PBS). Total RNA was extracted using Trizol reagent and reverse transcribed. Briefly, 2 μg RNA was used for reverse transcription under the following reaction

conditions: 42 °C for 15 min; 95 °C for 15 min and 0–5 °C for 5 min. PCR reaction was performed using 1 μl of cDNA with gene-specific primers in a 20 μl reaction volume. The thermocycle protocol included one pre-denaturation step at 95 °C for 5 min, followed by 35 amplification cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, and extension at 70 °C for 50 s, and then final extension at 72 °C for 7 min. As internal control, GAPDH (5′-CAACT ACATG GTTTA CATGT TC-3′, 5′-GCCAG TGGAC TCACG AC-3′, 180 bp) was amplified simultaneously. Products were analysed using 1.5% agarose gel electrophoresis.

Immunoblotting analysis. Transfected A549 cells were centrifuged at 13 000 g for 10 min at 4 °C. Cell lysates were prepared by resuspending cell pellets in lysis buffer containing 20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 mM DTT, and 1 μg/ml each of leupeptin and aprotinin. Equal amounts of protein were loaded on to SDS–PAGE gels, electrophoretically separated, and transferred to nitrocellulose membranes (Millipore, Bedlford, MA 01730, USA) using standard procedures. Membranes were blocked with 5% non-fat milk then probed with mouse monoclonal anti-hId3 (1:1000 dilution; Abcam, HKSP, Shatin, NT, Hong Kong) or rabbit anti-β-actin (1:800 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Membranes were then washed and incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG. Antibody binding was detected using an enhanced chemiluminescence detection system (Millipore).

Confocal fluorescence microscopy. Cells were grown on coverslips in 24-well plates. They were then transfected for 12–48 h in serum-free DMEM using Lipofectamine 2000. Afterwards, cultures were washed three times in PBS and fixed in acetone for 10 min at 4 °C. After washing twice in PBS, EGFP-expressing cells were detected using confocal fluorescence microscopy.

Flow cytometric analysis. Cells were transfected with pEGFP or pEGFP/Id3 for 12–96 h, harvested after trypsinization, washed three times in PBS, and stained with propidium iodide (PI). Numbers of EGFP-positive cells was then assessed by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Immunocytochemical analysis. Cells were maintained on coverslips in 24-well plates and transfected for 24 h. Transfected cells were washed three times in PBS and fixed using acetone at 4 °C for 10 min. After blocking with 10 g/l BSA for 2 h at 37 °C, fixed cells were incubated in mouse anti-hId3 antibody (1:500; Abcam Ltd.), for 2 h at room temperature. After rinsing in PBS, cells were stained with HRP-conjugated goat anti-mouse IgG (1:800; Boehringer Mannheim, Germany). Coverslips were rinsed in PBS, and antibody staining was visualized by five minute incubation in PBS containing 0.05% 3, 3-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. To confirm primary antibody specificity, anti-hId3 was pre-incubated with 5 μg/ml purified recombinant hId3 for 2 h at 37 °C before immunocytochemical staining.

Apoptosis

AnnexinV/7-AAD staining and flow cytometry. Apoptosis in cells transfected with pEGFP/Id3 or pEGFP (control group) was quantified using flow cytometry. Cells were harvested and pooled after 48 h transfection and stained with Annexin V-PE (BD Biosciences Pharmingen), a marker of early phase apoptosis, and with 7-aminoactinomycin (7-AAD) (BD Biosciences Pharmingen), an indicator of late apoptosis. Briefly, cells were washed twice in PBS and suspended in binding buffer containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. Staining mixture of 5 μl Annexin V-PE and 5 μl 7-AAD was added to 100 μl of cell suspension (1×10^6 cells/ml). Cells were incubated in the dark for 15 min at room temperature and then analysed on a FACSCalibur flow cytometer. EGFP-positive cells were sorted and analysed for DNA content. Percentage of apoptotic cells that bound Annexin V-PE but excluded 7-AAD was determined in the EGFP-positive cell population, to estimate fraction of Id3-expressing A549 cells in the early stages of apoptosis. Late apoptotic/necrotic cells were quantified by dual annexin V-PE and 7-AAD staining.

Hoechst 33258 staining. Apoptotic cells were also examined after nuclear staining using Hoechst 33258 staining kit (Beyotime, Haimen, China). Briefly, cell cultures were grown on coverslips in 24-well plates. Forty-eight hours after transfection, cells were washed twice in PBS and were fixed in acetone at room temperature for 2 h. Following second rinsing in PBS, fixed cells were stained with 0.5 ml Hoechst 33258 solution (167 μM) in the dark for 5 min. Finally, coverslips were rinsed in PBS and observed using a confocal fluorescence microscope.

Statistical analysis

Data from each treatment group were expressed as means ± SD. Means were compared using Student's

t-tests, and $P < 0.05$ was accepted as statistically significant.

Results

Construction of eukaryotic expression vector pEGFP/Id3

The cDNA encoding hId3 was subcloned into pEGFP vector to form recombinant eukaryotic expression vector pEGFP/Id3. Results of both restrictive enzymatic digestions (*Eco*R I and *Bam*H I) of pEGFP/Id3 and PCR identification, in which pEGFP/Id3 was used as template, showed a specific single band with the same molecular weight as reported for hId3 on agarose gel. DNA sequence analysis also indicated that the recombinant vector had been constructed successfully.

Id3-EGFP expression in A549 cells

After transfection, A549 cells were harvested and assayed for EGFP expression using confocal fluorescence microscopy and FCM. Eukaryotic expression vector pEGFP/Id3 vector contained a bicistronic mRNA encompassing *Id3* and *EGFP* genes under the control of the retroviral LTR promoter. Thus, expression of Id3 could be monitored by determining EGFP expression in transfected A549 cells. Cells were transfected with either pEGFP/Id3 or pEGFP for 12–96 h. Number of cells overexpressing Id3 was determined by counting number of EGFP-positive cells in FCM (Fig. 1a) or under confocal fluorescence microscopy (Fig. 1b). There was modest reduction in EGFP expression in pEGFP/Id3-transfected cells (33.69%) compared to pEGFP-transfected cells (41.07%) after 24 h, suggesting inhibition of proliferation. In addition, RT-PCR, western blotting, and immunocytochemistry revealed Id3 mRNA transcription and protein expression were significantly higher in pEGFP/Id3-transfected cells than in pEGFP-transfected control cultures (Fig. 2A–C).

Id3 expression inhibited proliferation of A549 cells

Effects of Id3 expression on cell proliferation and cell survival were then examined. During 96 h transfection with pEGFP, the fraction of EGFP-positive cells peaked slightly above 60% and remained stable for the next 2 days (Fig. 3). In cultures transfected with pEGFP/Id3, however, the fraction of EGFP-positive cells peaked at around 60% after 48 h and then began to decline drastically (Fig. 3). Cells expressing EGFP were almost undetectable after 96 h transfection, indicating that Id3

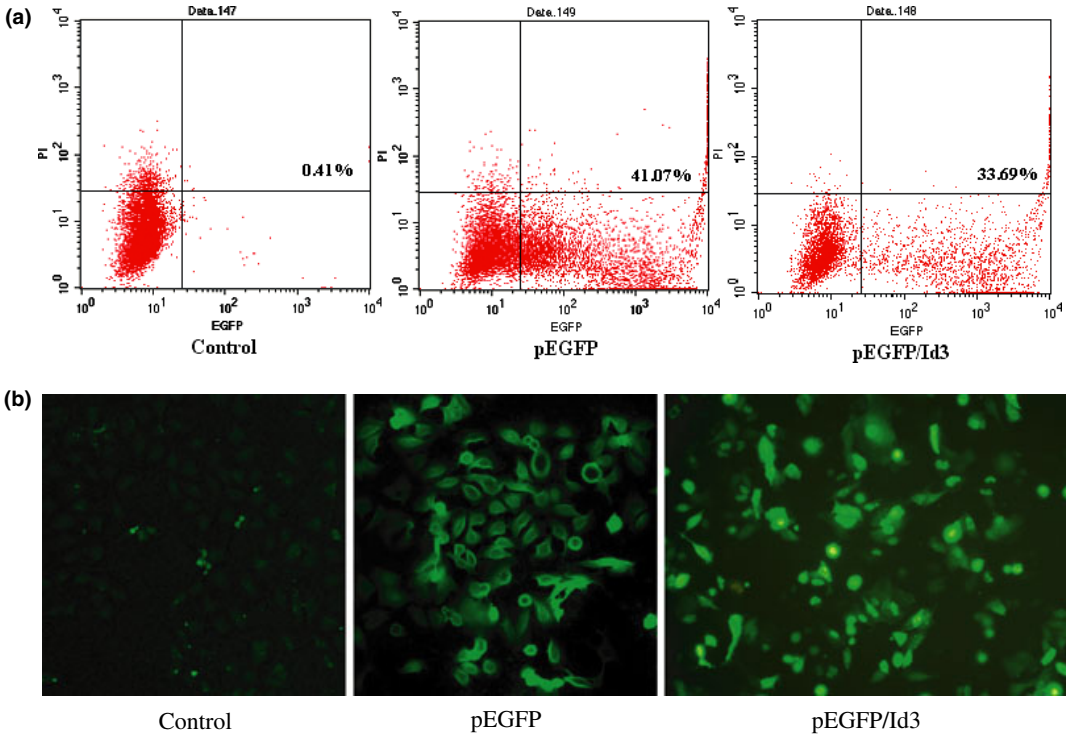

Figure 1. Expression of Id3-EGFP fusion protein in A549 cells. A549 cells were transfected with pEGFP and pEGFP/Id3 by Lipofectamine 2000. (a) Following 24 h transfection, cells were harvested and EGFP expression efficiency was assayed by flow cytometry (FCM). (b) For fluorescence microscopy, A549 cells were maintained on coverslips in 24-well plates and transfected with plasmids for 24 h. Coverslips were then fixed and EGFP-expressing cells were examined. In both (a) and (b) experiments, data from one of three or more representative experiments are shown.

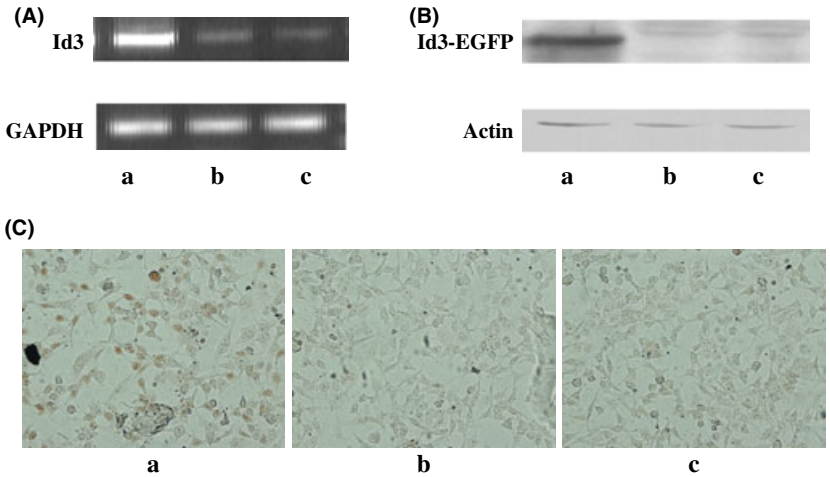

Figure 2. Ectopic overexpression of Id3 in A549 cells. Cells were transfected with pEGFP or pEGFP/Id3 for 48 h. Expression levels of Id3 mRNA and protein in transfected cells were determined by RT-PCR (A) and western blotting (B). (A) Total RNA was extracted and subjected to RT-PCR analysis with primers specific for Id3 and GAPDH as described in the Materials and methods section. (B) Cell lysates were prepared and subjected to western blotting analysis with mouse anti-hId3 antibody or rabbit anti-β-actin antibody as described in the Materials and methods section. (C) Intracellular distribution of ectopic Id3 expression was evaluated by immunocytochemical staining. After 48 h transfection, coverslips were washed, cells fixed, blocked and incubated with mouse anti-human Id3 antibody and HRP-conjugated goat anti-mouse IgG. Colour of final reaction product was developed and cells were observed by light microscopy (320×). (a) A549 cells transfected with pEGFP/Id3, (b) A549 cells transfected with pEGFP, (c) untreated A549 cells.

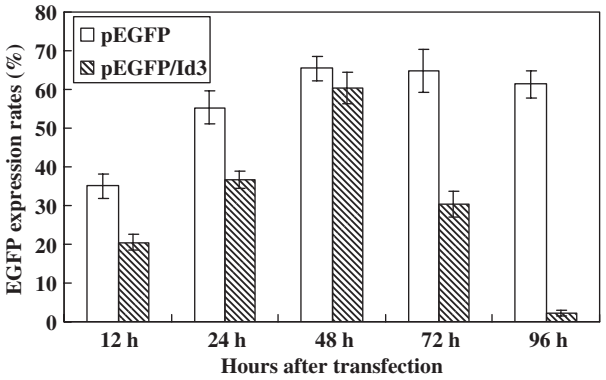

Figure 3. EGFP expression efficiencies at different time points after transfection. Cells were transfected with pEGFP or pEGFP/Id3 by Lipofectamine 2000. At 12–96 h after transfection, cells were harvested after trypsinization, washed in PBS and stained with PI. EGFP-positive cells were then monitored using a FACSCalibur flow cytometer. Results are presented as the means ± SD. from three independent experiments. $*P < 0.05$, $**P < 0.001$. Dramatic decline in EGFP-positive cells during transfection with pEGFP/Id3 indicated that Id3 expression inhibited proliferation or survival of A549 cells.

overexpression inhibited cell proliferation or promoted cell death.

Id3 induced apoptosis

To clarify the mechanism of cell proliferation inhibition induced by Id3 overexpression, apoptosis was examined by FCM following AnnexinV-PE and 7-ADD staining. Fractions of AnnexinV-positive cells in pEGFP/Id3-transfected cultures were significantly higher than in untransfected and pEGFP-transfected cultures (Fig. 4a). Apoptosis induced by ectopic Id3 overexpression was also evaluated by Hoechst 33258 staining. Confocal fluorescence imaging revealed significantly more cells with typical morphological characteristics of apoptosis, in cultures transfected with pEGFP/Id3 than in cultures expressing EGFP only (Fig. 4b).

Discussion

Inhibitor of differentiation (*Id*) genes are expressed in many tumour cell types, but complexity of their expression profiles has impeded functional analysis. In some cases, Id expression is a prognostic indicator; indeed, Id1 expression levels have been correlated with poor prognosis and shorter patient survival in some cancers (1–4). In contrast, there was consistent relationship found between Id3 expression profile and disease progression in other cancer types. Id3 isoform has been shown to be highly expressed in primary colorectal cancer and squamous cell carcinoma, while its expression in thyroid cancer and ovarian cancer seemed to be lower than in appropriate non-malignant tissues (22,23). Furthermore, Id3 appears developmentally regulated in some tumour cells, but not in others. Expression of Id3 in well-differentiated hepatocellular carcinoma cells (HCC) has been shown to be higher than in advanced dedifferentiated HCC, while Id3 was expressed at relatively constant levels during breast, prostate, and colon carcinogenesis (24). Our earlier

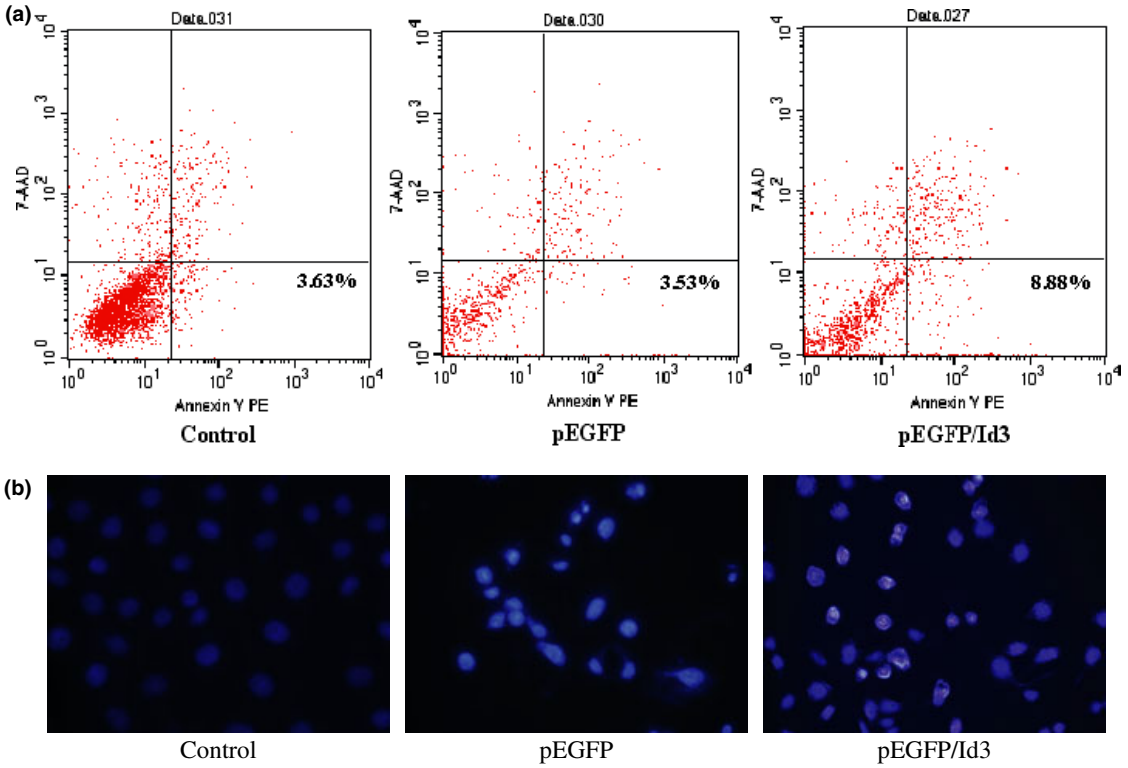

Figure 4. Overexpression of Id3 induces apoptosis. (a) A549 cells transfected with pEGFP or pEGFP/Id3 for 48 h were washed in PBS, suspended in binding buffer containing Annexin V and 7-AAD, and analysed by flow cytometry. (b) Apoptotic cells were also detected by nuclear staining with Hoechst 33258. Cells were maintained on coverslips in 24-well plates. Forty-eight hours after transfection, they were stained with Hoechst 33258 (167 μM) in the dark for 5 min. Nuclear morphology was examined using fluorescence microscopy. For both experiments (a) and (b), data from one of three or more representative runs are shown.

experimental results have demonstrated that Id3 was expressed at low levels in human lung cancer cell line A549; thus, we chose this cell type to examine effects of ectopic Id3 overexpression on cancer cell proliferation and survival.

Lung cancer remains at epidemic proportions and continues to be the leading cause of cancer death in both men and women. Despite new diagnostic and therapeutic techniques, most lung cancers are still detected late, and estimated 5-year survival rate for non-small cell lung cancer is below 18% (small cell lung cancer survival is below 7%). Thus, development of more efficacious therapies is clearly imperative. We constructed a eukaryotic expression plasmid, pEGFP/Id3, to induce ectopic overexpression of Id3 in A549 cells. High rates of EGFP expression were achieved after 48 h pEGFP/Id3 or pEGFP transfection (approximately 60%). The percentage of EGFP-positive cells remained stable in pEGFP-transfected cultures, but declined rapidly in cultures transfected with pEGFP-Id3; EGFP-positive cells were almost undetectable after 96 h pEGFP/Id3 transfection. To determine whether this reduction in EGFP/Id3-expressing cells was caused by apoptosis, we examined binding of apoptosis markers annexinV, 7-AAD and Hoechst 33258. Cells transfected with pEGFP/Id3 exhibited higher annexin V binding (8.88%) than cells transfected with pEGFP (3.53%). Nuclear imaging of pEGFP- and pEGFP/Id3-transfected cells using Hoechst 33258 stain mirrored these flow cytometry results, with many more pEGFP/Id3-transfected A549 cells exhibiting typical morphological features of apoptosis. Thus, overexpression of Id3 protein inhibited cell population growth by inducing apoptosis of A549 cells, confirming that Id3 expression could activate apoptotic pathways in human cancer cells as well as in B lymphocyte, keratinocytes and B lymphocyte progenitors (9).

Inhibitor of differentiation 3 has been implicated in apoptosis in response to cisplatin, a DNA-damaging agent. Cisplatin upregulated Id3 mRNA in MG-63 sarcoma cells, while ectopic expression of Id3 sensitized these sarcoma cells to cisplatin-induced caspase-3 activation and population growth inhibition (25). Subsequent studies have greatly illuminated molecular mechanisms regulating Id3 expression and Id3-mediated cell death.

Simbulan-Rosenthal and colleagues demonstrated that ectopic expression of Id3 induced apoptosis in immortalized KCs and that Id3 was strongly upregulated at both mRNA and protein levels when KCs were exposed to UVB radiation (19). In UVB-treated keratinocytes, apoptosis followed formation of Bax oligomers in the mitochondrial membrane, as determined by colocalization of Id3 and active Bax. In addition, ectopic expression of Bcl-2 blocked mitochondrial localization and activation of Bax, indicating that UVB triggered the mitochondria-dependent apoptotic pathway. Overexpression of Id3 also correlated with activation of caspases-3/9 within individual cells. These authors also found that Id3 mRNA was induced by UVB through generation of reactive oxygen species. Deletion of the *bax* promoter and mutational analyses revealed that a 738-bp region upstream of the transcription start site of *bax* was induced by ectopic expression of Id3 as well as by UVB, confirming the importance of UVB-mediated Id3 upregulation in activating pro-apoptotic *bax* promoter (20). In lymphocyte progenitors, however, Kee found that Id3-induced apoptosis occurred in the absence of p53 and was not inhibited by Bcl-2. Overexpression of Id3 promoted activation of caspase-2, caspase-3, and caspase-9, but only caspase-2 was required for Id3-induced apoptosis (18), suggesting that Id3 can also activate mitochondria-independent apoptosis.

In conclusion, we developed a eurkaryotic expression vector that allowed for ectopic overexpression of the putative pro-apoptotic and anti-tumour protein Id3 in human A549 lung cancer cells. Overexpression of Id3 inhibited cell proliferation and increased levels of apoptosis, while the same vector encoding EGFP alone had no effect on apoptosis relative to untransfected cells. These Id3-induced pro-apoptotic signalling pathways require further study, but this preliminary investigation suggests that Id3 regulation could be exploited in anti-tumour therapies.

Acknowledgements

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