Evidence of a novel antiapoptotic factor: Role of inhibitor of differentiation or DNA binding (Id-1) in anticancer drug-induced apoptosis

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Id-1 (inhibitor of differentiation or DNA binding), a member of the basic helix-loop-helix transcription factor family, is up-regulated in many types of human cancer and its expression levels are correlated with poor treatment outcome and shorter survival. In this study, we provided evidence to suggest that Id-1 is a universal survival factor that plays a key role in protection against anticancer drug-induced apoptosis. Using nine anticancer drugs and five cancer cell lines derived from nasopharyngeal carcinoma (CNE1), cervical carcinoma (HeLa), breast cancer (MCF7), hepatocarcinoma (Huh7) and prostate cancer (PC3), we found that down-regulation of Id-1 expression at both transcriptional and protein levels was associated with increased apoptosis rates and increased cleaved PARP after exposure to all anticancer agents. Treatment with a caspase 9 inhibitor, Z-LEHD-FMK, protected cancer cells from drug-induced PARP cleavage. However, overexpression of Id-1 in a p53 mutated cell line, CNE1, was able to suppress PARP cleavage in response to all anticancer drugs examined. In contrast, down-regulation of Id-1 through small RNA technology in CNE1 cells led to increased sensitivity to all six types of chemotherapeutic drugs. Our results demonstrate that Id-1 may be a general negative regulator of anticancer drug-induced apoptosis and suggest a novel therapeutic target in inducing chemosensitization in cancer cells. Our evidence also provides a possible underlying mechanism responsible for the positive role of Id-1 in the progression of human cancer. (*Cancer Sci* 2007; 98: 308–314)

Id-1 (inhibitor of differentiation or DNA binding), a member of the basic helix-loop-helix (HLH) transcription factor family, has been suggested as a potential oncogene. This is because overexpression of Id-1 in normal cells is able to promote cell proliferation through the inactivation of tumor suppressor pathways such as p16INK4a/RB.[1,2] In addition, up-regulation of Id-1 has been reported in more than 20 types of human cancer, most of which are of epithelial origin, including breast,[3] pancreas,[4] cervical,[5] head and neck,[6] and prostate cancers.[7] Id-1 expression levels are also positively correlated with advanced tumor stage as well as poor prognosis in several types of human cancer.[4,5,7] Furthermore, recently it has been reported that patients with higher levels of Id-1 expression have much shorter overall survival in ovarian cancer,[8] melanoma,[9] and breast cancer.[10] Up-regulation of Id-1 in early stage cervical cancer has been suggested as a marker for poor prognosis.[5] These results indicate that overexpression of Id-1 may play an important part not only in tumorigenesis but also in the progression of human cancer. This hypothesis is also confirmed by several cDNA array studies demonstrating that up-regulation of Id-1 is one of the most frequent events during the development of tumorigenesis in animal models.[11–13] Recently, inactivation of Id-1 is reported to have a therapeutic potential. For example, in cultured prostate cancer cells, down-regulation of Id-1 by siRNA technology leads to chemosensitization to a commonly used anticancer drug, taxol.[14] Furthermore, in a breast cancer animal model, inactivation of Id-1 is able to suppress the metastatic spread of cancer cells to the lung.[12] These lines of evidence strongly indicate Id-1 as a promising therapeutic target for the treatment of human cancer.

Although the precise molecular mechanisms responsible for its cancer promoting action are not well understood, recent evidence suggests that high levels of Id-1 expression in cancer cells might lead to suppression of the apoptosis pathway, resulting in promotion of cell survival and cancer progression. Several lines of evidence support this hypothesis. First, overexpression of Id-1 confers resistance to TNF-α-induced apoptosis in prostate cancer cells through activation of the nuclear factor (NF)-κB pathway.[15] In addition, ectopic expression of Id-1 leads to resistance to an anticancer drug, taxol, through suppression of the apoptosis in nasopharyngeal carcinoma (NPC) cells.[16] Furthermore, overexpression of Id-1 results in acquired drug resistance to doxorubicin in androgen independent prostate cancer cells.[17] In contrast, suppression of Id-1 gene expression through small RNA technology leads to hypersensitivity to taxol-induced apoptosis in prostate cancer cells.[14] In addition, in gastric cancer cells, down-regulation of Id-1 is associated with the nonsteroidal anti-inflammatory drug sulindac sulfide induced cell death.[18] In melanoma cells, Id-1 was greatly reduced when cells were undergoing *All-trans*-retinoid acid-induced apoptosis.[19] Based on these lines of evidence we hypothesized that Id-1 might be a common antiapoptotic factor and high levels of Id-1 in cancer cells could act as protection against a variety of anticancer drug-induced cell death. To test this hypothesis, using six types of anticancer agents with different mechanisms of action, we treated five cancer cell lines derived from five types of human cancer, all of which showed an up-regulation of Id-1 expression in clinical specimens,[3,5,7,20,21] and studied the role of Id-1 in anticancer drug-induced apoptosis. Our results suggest that Id-1 is a novel universal antiapoptotic factor that may play a key role in the protection of anticancer drug-induced apoptosis.

Materials and Methods

Anticancer agents. Two DNA damaging agents (cisplatin, mechlorethamine), one antibiotic (mitomycin C), two topoisomerase II inhibitors (doxorubicin, etoposide), one antimetabolite (methotrexate), two microtubule disrupting agents (taxol, vincristine), and a green tea extract, epigallocatechin gallate (EGCG) were purchased from Calbiochem, CA, USA except for mechlorethamine (Sigma, MO, USA). Taxol and etoposide were diluted in dimethylsulfoxide, while mitomycin C was diluted with hot phosphate-buffered saline (PBS), and the rest of the drugs were all diluted in PBS. The stock solutions of each drug were further diluted again in culture medium to obtain the desired concentrations. Table S1 lists the summary of drug concentrations used in this study.

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Cell lines and cell culture conditions. CNE1 (nasopharyngeal carcinoma cell line),[22] HeLa (cervical cancer cell line), MCF7 (breast cancer cell line), Huh7 (hepatocarcinoma cell line) and PC3 (androgen-independent prostate cancer cell line) obtained from American Type Culture Collection (Rockville, MD, USA), were maintained in RPMI 1640 medium (Sigma) supplemented with 5% (v/v) fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL) at 37°C, 5% CO_2, except for Huh7 which was cultured in Dulbecco's modified Eagle's medium containing 10% FBS. A stable Id-1 transfectant cell line and the vector control generated from CNE1 cells (CNE1 pBabe and CNE1 Id-1) in a previous study[21] were also included.

Terminal deoxynucleotidyl transferase fluorescence-dUTP nick end labeling (TUNEL) assay. Using an *in situ* cell death detection kit, fluorescein was used to detect apoptotic cells according to the recommended protocol (Roche Dignostics). Briefly, cells were seeded on 4-mm well diameter Chamber slides (ICN, Biomedicals, Aurora, OH, USA) and 24 h later the cells were treated with drugs with or without Caspase 9 inhibitor Z-LEHD-FMK (Calbiochem). They were fixed with 4% paraformaldehyde and incubated with reagents supplied in the kit after the permeabilization process. Then propidium iodide (10 μg/mL in PBS) was added to visualize cell nucleus. The percentage of TUNEL positive cells was calculated under a fluorescent microscope. At least 500 cells were counted in each experiment and the percentage of apoptotic cells was calculated as the number of apoptotic cells over the total number of cells counted × 100.

MTT assay. Cell viability was measured using 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) proliferation assay kit (Boeringher, MO, USA), and the experimental procedures were described previously.[14] Briefly, 3000–6000 cells were seeded in 96-well plates and cultured for 24 h, drugs (dose 3) were added, respectively. Cell viability was examined 24 h after treatment.

Western blotting. Detailed experimental procedures were described previously.[23] Briefly, whole-cell lysate was prepared by resuspending cell pellets in lysis buffer, and protein concentrations were measured using the protein assay kit (Bio-Rad, Hercules, CA, USA). Protein suspension (15–30 μg) was then loaded onto sodium dodecylsulfate–polyacrylamide gel electrophoresis for electrophoresis and then transferred to a polyvinylidene difluoride membrane (Amersham, Piscataway, NJ, USA). The membrane was then incubated with primary antibodies for 1–2 h at room temperature against Id-1(Santa Cruz Biotechnology, CA, USA), Bax, PARP (Cell Signaling Technology, MA, USA) and β-actin (Santa Cruz Biotechnology), respectively. After washing with Tris-buffered saline Tween-20 (TBS-T), the membrane was incubated with a secondary antibody against rabbit or goat IgG and the signals were visualized using the enhanced chemiluminescence plus Western blotting system (Amersham).

Reverse transcription-polymerase chain reaction. Total RNA was isolated from drug-treated and control cells using a Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). For reverse transcription-polymerase chain reaction (RT-PCR), cDNAs were synthesized using the SuperScript First Strand Synthesis System (Invitrogen). The cDNA was then amplified by PCR with Id-1 specific primers (sense: 5′-CCG GCA AGA CAG CGA GCG GTG CG-3′; antisense: 5′-GGC GCT GAT CTC GCC GTT GAG GG-3′) as described.[24] The PCR conditions were as follows: an initial denaturation at 95°C was followed by 28 cycles of PCR (94°C for 1 min, 58°C for 1 min and 72°C for 1 min), and a final extension at 72°C for 5 min. The *GAPDH* was amplified as an internal loading control. PCR products were electrophoresed on 1.5% agarose gels and analyzed using a gel documentation system (Ultra-Violet Product Limited, CA, USA).

Transient si-RNA transfection. siGENOME siRNA targeted the *Id-1* gene. It was purchased commercially (Dharmacon, CO,

USA) and dissolved in RNase-free distilled water. Cells were seeded and cultured overnight. Then the si-genome duplex RNA (60 nM) (Si-Id-1: Sense: UAAACGUGCUGCUCUACGA, Antisense: UCGUAGAGCACGUUUA; Si-Id-1-2: Sense: GGACGAGCAG-CAGGUAAAC; Antisense: GUUUACCUGCUGCUCGUCC) and si-CON (60 nM) (Sense: UAGCGACUAAACACAUCAA, Antisense: UUGAUGUGUUUAGUCGCUA) was mixed, respectively, with lipofectamine 2000 (Invitrogen), then added into the culture medium. The cells were collected after 48 h, or treated with drugs after 24 h and then collected after 48 h.

Results

Down-regulation of Id-1 is correlated with increased anticancer drug-induced apoptosis and decreased cell viability. To test the hypothesis that Id-1 might play a positive role in protecting cancer cells from programmed cell death, we studied the role of Id-1 on five cancer cell lines derived from nasopharyngeal carcinoma (CNE1), cervical carcinoma (HeLa), breast cancer (MCF7), hepatocarcinoma (Huh7) and prostate cancer (PC3), all of which have been shown to overexpress Id-1 in clinical specimens.[3,5,7,20,21] Nine commonly used anticancer drugs including two DNA damaging agents (cisplatin, mechlorethamine), one antibiotic (mitomycin C), two topoisomerase II inhibitors (doxorubicin, etoposide), one antimetabolite (methotrexate) and two microtubule disrupting agents (taxol, vincristine), were used to examine the association between Id-1 expression and the anticancer drug-induced apoptosis. In addition, a green tea extract, epigallocatechin gallate (EGCG), which has been shown to induce apoptosis in cancer cells through multiple pathways,[25] was also included as a representative for alternative anticancer agents. As shown in Fig. 1, after exposure to the anticancer agents (summary of drug doses is shown in Table S1), all of the cell lines showed a dose-dependent down-regulation of the Id-1 protein. In addition, RT-PCR experiments showed that the decreased Id-1 protein expression was associated with decreased Id-1 mRNA level in response to the anticancer drugs (Fig. S1). However, the expression of Bax, a proapoptotic factor, was not significantly altered by any of the treatments, indicating that down-regulation of Id-1 may not be a result of overall transcriptional suppression of the drug treatment. In contrast, the expression of cleaved PARP, an indicator of activation of the apoptosis pathway, was increased (see arrows). The increased PARP cleavage was associated with increased TUNEL positive cells (Fig. 2) and decreased cell viability (Fig. 1b). These results suggest that decreased Id-1 expression is correlated with the activation of apoptosis pathway and apoptotic cell death in human cancer cells in response to all nine anticancer drugs.

To further confirm these results, we then treated the cells with Z-LEHD-FMK, a caspase 9 inhibitor, which has been shown to suppress the apoptosis pathway.[26] As shown in Fig. 3, the expression of cleaved PARP was decreased in the cells treated with both anticancer drugs and Z-LEHD-FMK compared to the cells treated with drugs alone (Fig. 3a). The decreased PARP cleavage was also associated with a decreased percentage of TUNEL positive cells in the Z-LEHD-FMK treated cells (Fig. 3b). Treatment with a higher dose of Z-LEHD-FMK led to suppression of cleaved PARP levels but not the expression of Id-1 (Fig. 3c). Our data suggest that apoptosis might be the result of Id-1 downregulation by anticancer drug treatment, because in the absence of Z-LEHD-FMK, increased cleaved PARP was associated with decreased Id-1 expression after drug treatment. However, in the presence of Z-LEHD-FMK, an inhibitor of caspase 9, though the expression of Id-1 was decreased after drug treatment, cleavage of PARP was inhibited. These results suggest that Id-1 could be one of the upstream regulators of apoptosis. These results further support the suggestion that the down-regulation of Id-1 mediated chemosensitivity is
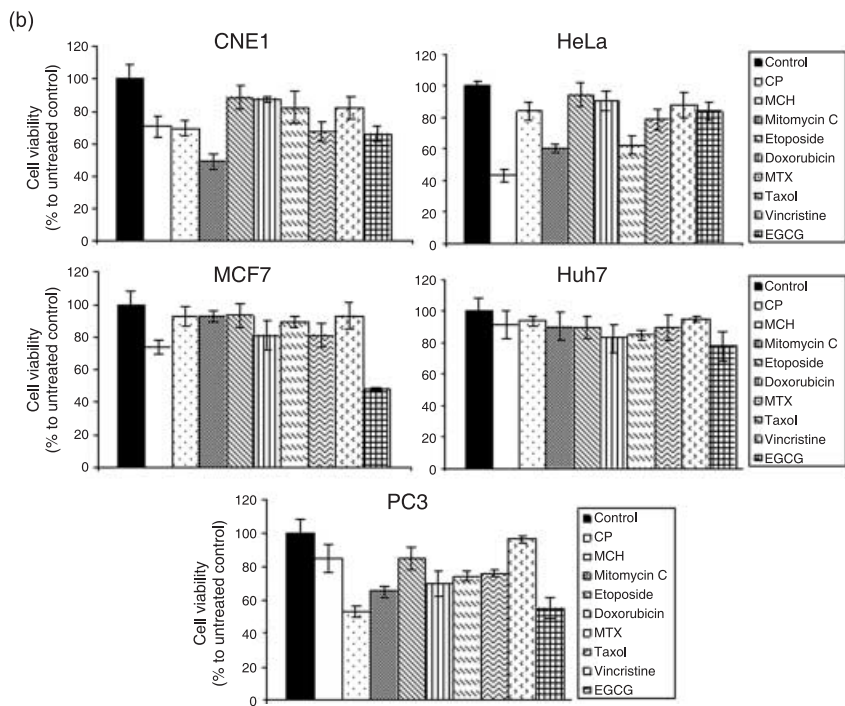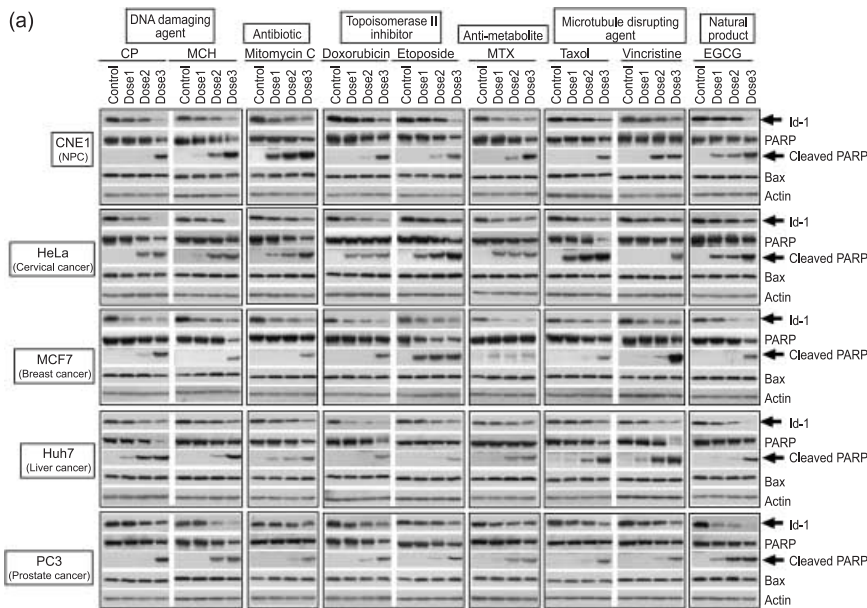
Fig. 1. Effect of anticancer drugs on Id-1, Bax and PARP expression and cell viability. (a) Western blotting analysis of Id-1, Bax and PARP expression in response to six types of anticancer drugs. Five cancer cell lines. CNE1 (NPC), HeLa (cervical cancer), MCF7 (breast cancer), Huh7 (hepatocarcinoma) and PC3 (prostate cancer), were exposed with three doses of each anticancer drug for 24 h, respectively, and protein lysates were analyzed. Note that decreased Id-1 is associated with increased cleaved PARP expression. (b) Cell viability of five cancer cell lines, after exposure to six types of anticancer drugs (dose 3) for 24 h, examined by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. CNE1, Hela, MCF7, Huh7 and PC3 cells were seeded in 96-well plates and drugs (dose 3) were added 24 h later. Cell viability was examined 24 h after treatment. Results represented the optical density (OD) ratio between the treated and untreated cells. Each data point represented the mean and standard deviation. Note that cell viability is decreased after drug treatment. CP, cisplatin; EGCG, epigallocatechin gallate; MCH, mechlorethamine; MTX, methotrexate.

through the apoptosis pathway and indicate that Id-1 could be an upstream regulator of the anticancer drug-induced apoptosis.

Overexpression of Id-1 suppresses anticancer drug-induced apoptosis. To investigate if overexpression of Id-1 could protect cancer cells from apoptosis against all nine anticancer drugs, we then treated two previously generated CNE1 sublines (pBabe and Id-1) with relatively high (CNE1 Id-1) and low (CNE1 pBabe)[21] Id-1 expression (Fig. 4a). As shown in Fig. 4(b), after treatment with the same doses of anticancer drugs, the pBabe cells showed much lower Id-1 protein levels (arrows) compared to the Id-1 transfectants in a dose dependent manner, although decreased Id-1 expression was observed in both cell lines. In addition, the relatively higher Id-1 protein levels in the Id-1 transfectants were associated with lower levels of cleaved PARP (see arrows) and decreased percentages of TUNEL positive cells (data not shown) and increased cell viability (Fig. 4c) after

exposure to all nine anticancer agents. These results indicate that high levels of Id-1 are able to protect cancer cells from apoptosis induced by a variety of anticancer drugs.

Down-regulation of Id-1 leads to increased sensitivity to anticancer drug-induced apoptosis. To further investigate the possibility of using Id-1 as a target to increase chemosensitivity in human cancer cells, we then suppressed Id-1 expression in CNE1 cells through small RNA interference and studied the effect of Id-1 suppression on anticancer drug-induced apoptosis. As shown in Fig. 5(a), Id-1 expression was much lower in the si-Id-1 transfectants (up to an 80% decrease) compared to the control cells, indicating a successful inhibition of Id-1 expression. Western blotting analysis showed that after exposure to nine anticancer drugs, respectively, the differential Id-1 expression between the si-Id-1 and the control cells was even greater in a dose-dependent manner (Fig. 5b). The decreased Id-1 protein expression was
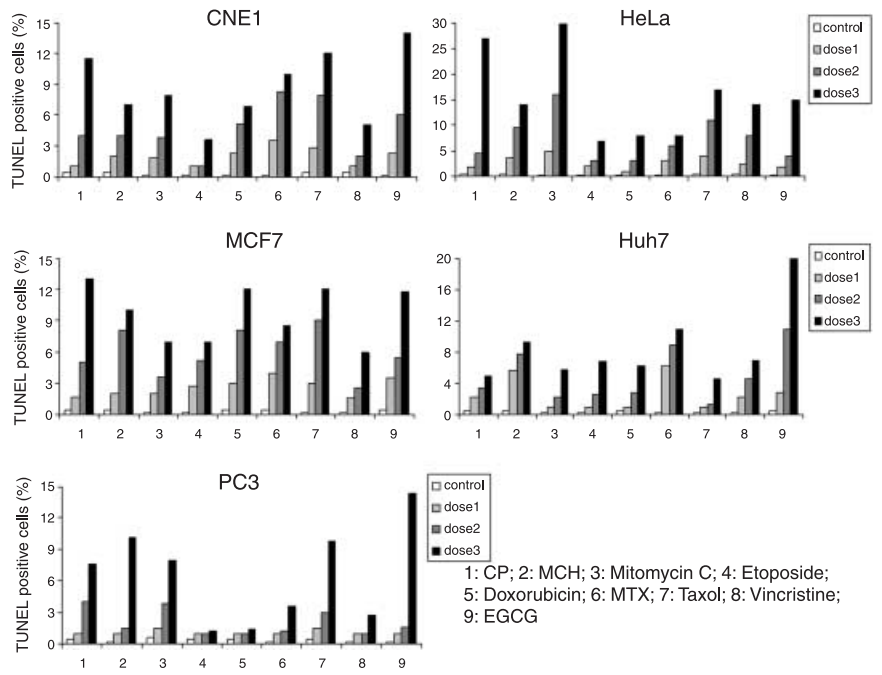
Fig. 2. Evidence of anticancer drug-induced apoptosis in cancer cells. Cells were treated with three doses of each anticancer drug for 24 h, respectively, and the percentage of TUNEL positive cells was determined. 1, cisplatin (CP); 2, mechlorethamine (MCH); 3, Mitomycin; 4, Etopside; 5, Doxorubicin; 6, methotrexate (MTX); 7, Taxol; 8, Vincristine; 9, epigallocatechin gallate (EGCG). Less than 1% TUNEL positive cells was observed in the untreated samples. Note that increased percentage of TUNEL positive cells is associated with increased drug concentration.
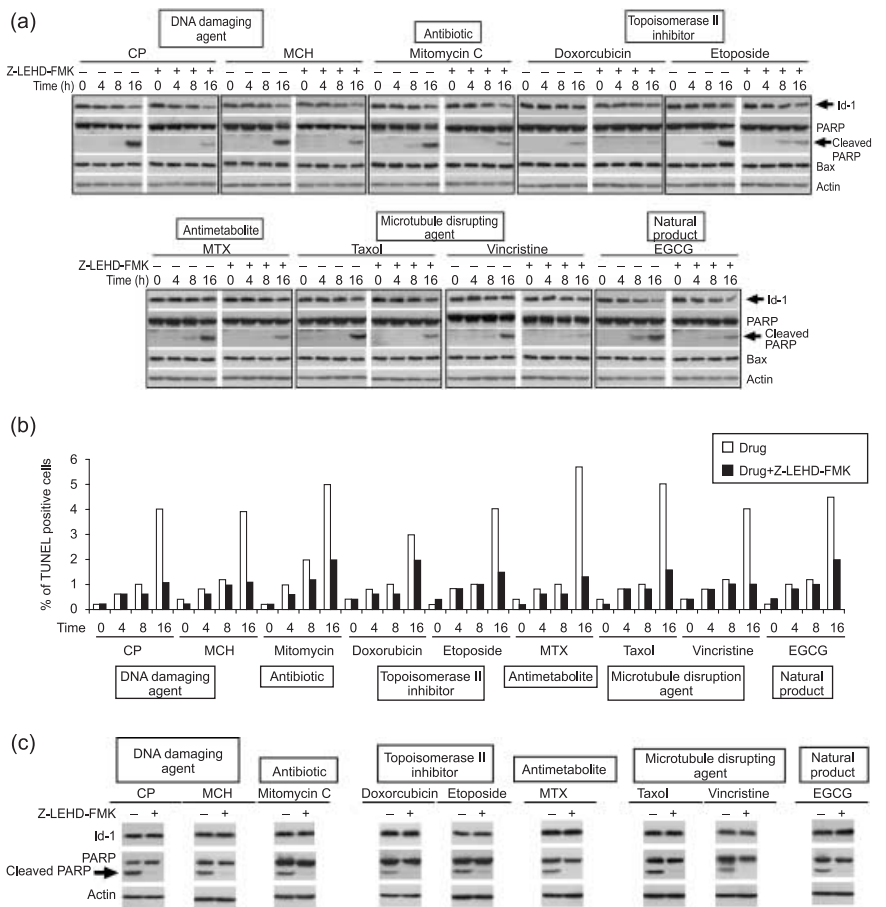

Fig. 3. Effect of Caspase 9 inhibitor on anticancer drug-induced apoptosis. (a) Western blotting analysis of Id-1, PARP and Bax expression of CNE1 with or without treatment with a caspase 9 inhibitor, Z-LEHD-FMK, 20 μM, and with or without drugs (dose 3). (b) Anticancer drug-induced apoptosis in response to Z-LEHD-FMK examined by TUNEL assay. (c) Effect of a higher dose of Z-LEHD-FMK (40 μM) on the expression of PARP and Id-1.

associated with higher levels of cleaved PARP in the si-Id-1-1 cells (Fig. 5b; arrows) as well as an increased percentage of TUNEL positive cells (data not shown). These results were confirmed in the cells transfected with another Si-Id-1 sequence

(Si-Id-1-2, Fig. 5c,d). These results further support the negative role of Id-1 in anticancer drug-induced apoptosis and suggest inactivation of Id-1 might be a target to induce chemosensitization to a variety of anticancer drugs.
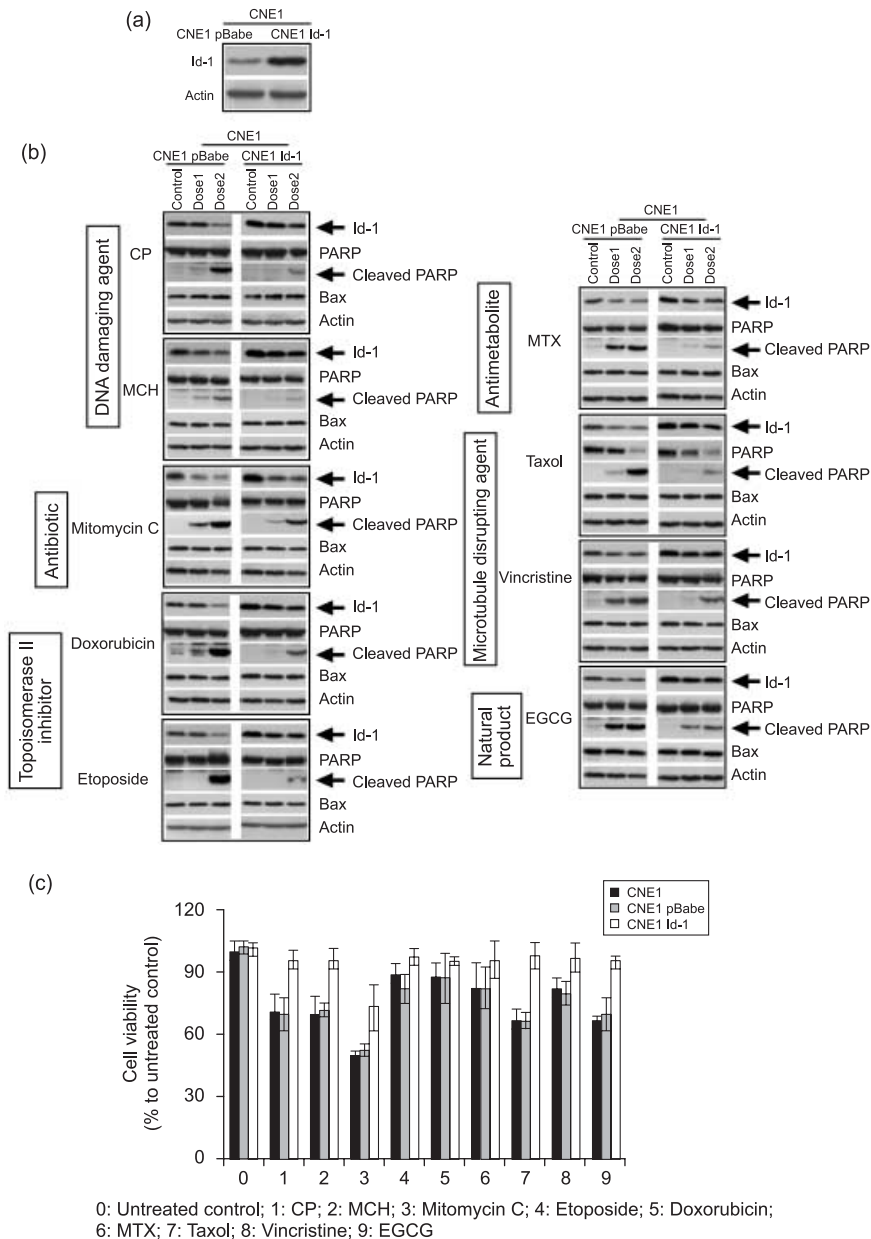
Fig. 4. Effect of Id-1 overexpression on anticancer drug-induced apoptosis. (a) Differential Id-1 expression between CNE1 pBabe and CNE1 Id-1 transfectants cultured in serum free medium. Stable transfectants were generated using an Id-1 expression vector as described. (b) Western blotting analysis of Id-1, PARP and Bax expression in response to anticancer drugs after 24 h treatment in the cells with high and low levels of Id-1 protein. (c) Cell viability of CNE1, CNE1 pBabe and CNE1 Id-1, after exposure to six types of anticancer drugs (dose3) for 24 h, examined by 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. CP, cisplatin; EGCG, epigallocatechin gallate; MCH, mechlorethamine; MTX, methotrexate.

Discussion

Overexpression of Id-1 has been reported in over 20 types of human cancer,[27] and its expression levels have been associated with tumor progression, poor prognosis and shorter survival.[5–10] In this study, we demonstrated that the presence of high levels of Id-1 protein in cancer cells is able to provide protection against activation of the apoptosis pathway by nine anticancer drugs. Since these agents induce cancer cell death through at least six different pathways, it is possible that Id-1 might be a common mediator of anticancer drug-induced apoptosis. Increased Id-1 expression in cancer cells might serve as a universal antiapoptotic factor leading to promotion of cell survival. In addition, our results that down-regulation of Id-1 leads to increased sensitivity to chemodrug-induced apoptosis implicate a novel molecular therapeutic target for inducing chemosensitization in human cancer cells.

As discussed, overexpression of Id-1 has been reported to confer resistance to certain anticancer drugs such as taxol,[14] TNF-α,[15] and doxorubicin.[17] However, the present study has provided the first evidence to demonstrate that the antiapoptotic effect of Id-1 was observed not only in a variety of cancer types, but also against six types of anticancer drugs. More importantly, our results could provide an explanation for the previous clinical studies on nasopharyngeal carcinoma, cervical carcinoma, breast cancer, hepatocarcinoma and prostate cancer as to why overexpression of Id-1 is correlated with disease progression in cancer patients.[3,5,7,20,21] In addition, our results also suggest a possible mechanism underlying the association of Id-1 with poor prognosis and shorter survival reported in several types of human cancer patients.[5–10] Targeting genes that are essential for cell survival has always been a key interest in the field of drug development. In this study, our results that suppression of Id-1 by small RNA interference led to increased sensitivity to nine anticancer drug-induced apoptosis (Fig. 5), suggest that down-regulation of Id-1 could be a therapeutic strategy to induce chemosensitization of conventional anticancer drugs in advanced cancers.

It has been well established that the p53-dependent pathways play a key role in mediating anticancer drug-induced apoptotic cell death.[28] Although a majority of studies suggest that the
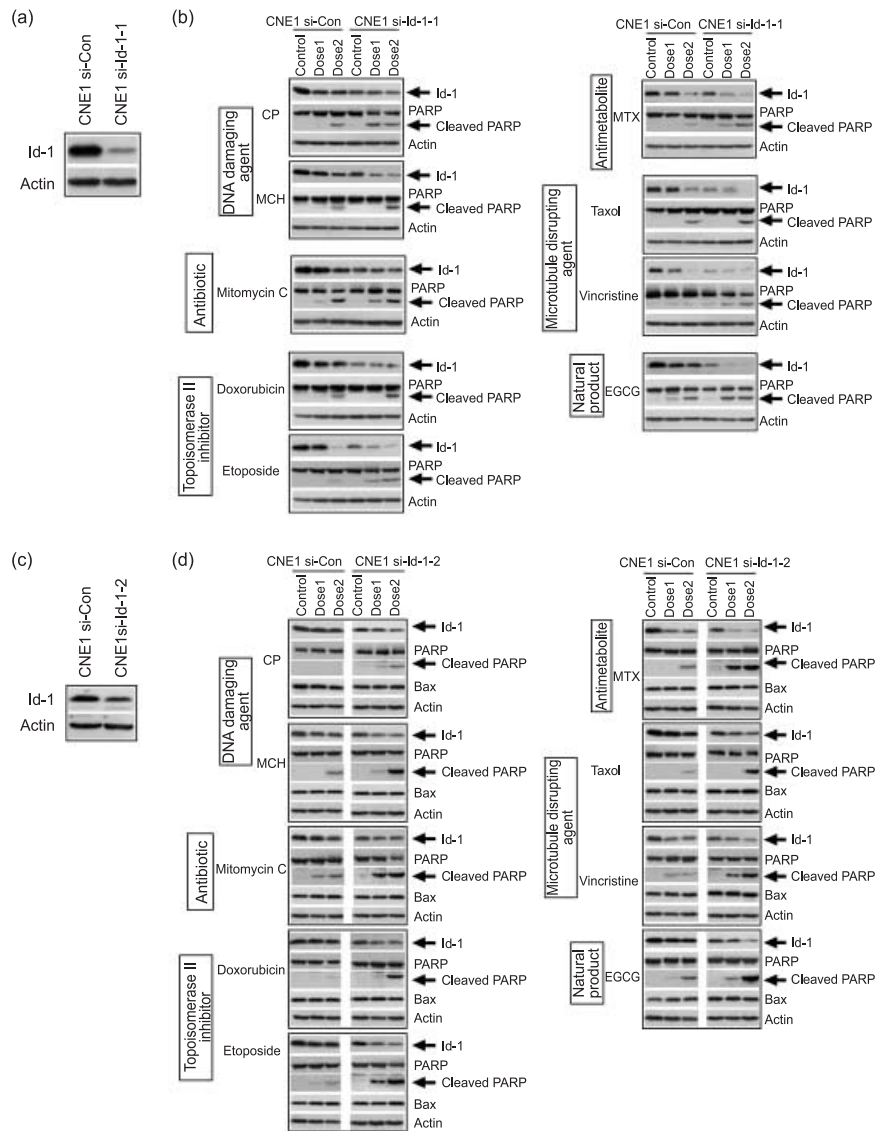
Fig. 5. Effect of Id-1 inactivation on anticancer drug-induced apoptosis. (a), (c) Differential Id-1 expression between CNE1 cells expressing high and low levels of Id-1. Two Si-RNA sequences targeted to Id-1 (Si-Id-1; Si-Id-1-2) and control sequence were transfected with Lipofectamine™ 2000 in CNE1 cells, respectively. (b), (d) Western blotting analysis of Id-1, PARP and Bax expression in response to anticancer drugs for 24 h.

oncogenic role of Id-1 is mainly mediated through p16INK4a/ RB pathway,[1,2] recently it was reported that mutational inactivation of p53 in a prostate cancer cell line, LNCaP, was able to induce Id-1 expression that could contribute to its increased proliferative potential.[29] However, in this study, although the five cancer cell lines examined contained differential p53 status from a functional wild type p53 (MCF7, HeLa),[30,31] a mutated p53 gene (CNE1, Huh7),[32,33] to a deleted p53 (PC3),[34] a consistent negative correlation between Id-1 expression and drug-induced apoptosis was observed. This suggests that the Id-1 mediated antiapoptotic effect could be independent of a p53 pathway. Interestingly however, the basal levels of Id-1 protein seemed to be higher in the p53 deleted PC3 cell line compared to the lines with a wild type p53 (Fig. 1). Although it is far from clear how Id-1 interacts with the apoptosis pathway in response to multiple extracellular stresses, or the molecular mechanisms responsible for its antiapoptotic effect, it is possible that it could be regulated through several signaling pathways. This hypothesis is strongly supported by the results demonstrated in this study that down-regulation of Id-1 was universally observed in response to a broad spectrum of anticancer drugs, which induce cancer cell death through diverse pathways from directly binding to DNA to microtubule disruption. In additional support to this argu-

ment, previous reports also showed that the Id-1-induced protection against apoptosis was mediated through various pathways including the NF-κB, c-Jun N-terminal kinase (JNK) and Raf/ mitogen-activated protein kinase kinase (MEK) pathways.[14–16] With these lines of evidence in mind and taking into account our new results, it is possible that like Bcl-2, Id-1 might be a novel universal survival factor whose main function is to protect cancer cells from extracellular stress-induced apoptosis. Overexpression of Id-1 in cancer cells could provide a survival advantage in response to chemodrug-induced apoptosis, therefore promoting drug resistance leading to cancer progression. Currently, we are investigating how Id-1 regulates the apoptosis cascade and what role it plays in mediating cellular responses to a variety of anticancer drugs. In conclusion, the current study demonstrates that Id-1 is a novel universal antiapoptotic factor, and provides the first evidence to suggest a therapeutic target for the management of advanced cancers.

Acknowledgments

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Supplementary Material

The following supplementary material is available for this article:

Fig. S1. RT-PCR analysis of Id-1 expression in CNE1 cells in response to anticancer drugs. Cells were treated with anticancer drugs for 24 h and Id-1 mRNA expression was analyzed. The relative intensity of the PCR product bands are shown as ratios to GAPDH underneath each gel. Note that Id-1 mRNA expression is decreased with increased drug concentrations.

Fig. S2. Representative photos of TUNEL results. CNE1 cells were treated with EGCG of three different doses for 24 h and the cells were fixed with 4% paraformaldehyde. TUNEL assay was performed using *in situ* cell death detection kit, Fluorescein.

Table S1. Summary of drug doses used for each treatment

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