Id-1-induced Raf/MEK pathway activation is essential for its protective role against taxol-induced apoptosis in nasopharyngeal carcinoma cells

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Increasingly, evidence supports the function of the helix–loop–helix protein Id-1 (inhibitor of differentiation/DNA binding-1) as an oncogene. Over-expression of Id-1 is not only observed in many types of human cancer but its expression levels have been correlated with cancer progression. However, little is known about the molecular mechanisms responsible for the function of Id-1. Recently, we and others reported that Id-1-induced cell proliferation was mediated through a Raf/MEK signalling pathway. In this study, we investigated if ectopic Id-1 expression in nasopharyngeal carcinoma cells had any protective effect on taxol-induced death, which is also regulated through Raf/MEK pathway. Using four stable Id-1 transfectant clones, we found that exogenous Id-1 expression led to phosphorylation of Raf-1 and MEK1/2 kinases, which was associated with resistance to taxol. Treatment of the Id-1 expressing cells with a MEK inhibitor, PD098059, resulted in an increased taxol-induced apoptosis rate in Id-1 transfectants compared with the vector control. In addition, the fact that the taxol-induced apoptosis rate, down-regulation of Bcl-2 and up-regulation of Bax were suppressed by PD098059 treatment in Id-1 expressing cells indicates that the Id-1 induced cellular protection against apoptosis is mediated through Raf/MEK signalling pathways. Our results suggest that Id-1 may be an upstream regulator of the Raf/MEK signalling pathway, which plays an essential role in protection against taxol-induced apoptosis. Our evidence also indicates a novel treatment strategy to increase anticancer drug-induced apoptosis through inactivation of the Id-1 protein.

Introduction

Id family proteins (inhibitor of differentiation/DNA binding) heterodimerize and act as dominant-negative regulators of helix–loop–helix transcription factors, thus inhibiting gene expression (1). Recent studies suggest that Id proteins, especially Id-1, may function as oncogenes. For example, Id-1 can stimulate DNA synthesis, initiate G1 to S phase transition (2–4) and extend the life span of primary human keratinocytes (5,6). In addition, up-regulation of Id-1 has been found in many types of human cancer such as breast (7), pancreatic (8), prostate (9) and ovarian (10) carcinomas. Recently, over-expression of Id-1 has been indicated as a marker for unfavourable prognosis in cervical and breast cancers (10,11) and the level of Id-1 protein expression is correlated with the more aggressive clinical behaviour of ovarian and prostate carcinomas (9,12). Furthermore, in Id-1+/−Id-3−/− knockout mice, a significant reduction in the metastatic ability of tumour xenografts is found (13). These lines of evidence indicate that expression of Id-1 protein may provide intracellular signals in favour of growth and survival in tumour cells.

Several signalling pathways have been suggested to mediate the oncogenic function of Id-1. For example, Id-1 has been shown to directly inhibit p16INK4a expression and induce phosphorylation of RB leading to increased cell proliferation (4,14). It is also reported that Id-1 facilitates human primary fibroblasts escaping senescence through a Ras/Raf/MEK pathway (15). Recently, we have found that in human prostate cancer cells, activation of Raf/MEK pathways is crucial for Id-1-induced serum-independent cell proliferation (16). The fact that targeted deletion of MEK1 in mice is lethal (17) indicates that Raf/MEK pathways may provide biological signals essential for cell survival. Since activation of factors involved in MAPK signalling pathways such as Raf and MEK, is a frequent event in certain types of human cancer (18,19), it is possible that activation of MAPK signalling pathways plays an important role in providing a survival advantage for tumour cells. In addition, increased expression of downstream targets of the Raf/MEK pathway such as Egr-1 and EGFR, is more commonly observed in aggressive and advanced tumours (20,21), which usually show resistance to clinical treatment, further indicating a protective role of the Raf/MEK pathway against cell death in tumour cells.

Previously, we reported that up-regulation of Id-1 protein was found in nasopharyngeal carcinoma (NPC) and ectopic expression of Id-1 in NPC cells led to increased cell proliferation (22). Since up-regulation of Egr-1 and EGFR is also observed in NPC (23–25) and recently we found that ectopic Id-1 expression was able to induce Egr-1 expression in prostate cancer cells (16), the aim of this study was to investigate if Id-1 played a part in the activation of the Raf/MEK signalling pathway in NPC cells and to determine whether Id-1-induced MAPK pathway activation could play a protective role against anticancer drug taxol-induced cell death in NPC cells.

Materials and methods

Cell lines

A NPC cell line, CNE1, derived from a poorly differentiated NPC patient (26), was transfected with an Id-1 expression vector (a gift from Dr P.Y.Desprez, Geraldine Brush Cancer Research Institute, California Pacific Medical Center) (7) and four stable transfectant clones were generated. Detailed experimental procedures on transfection and cloning of the transfectants were described previously (22). The cells were maintained in RPMI1640 (Sigma, St Louis, MO) supplemented with 2 mM l-glutamine and 5% (v/v) fetal calf serum at
As shown in Figure 1, all of the four Id-1 transfectants showed phosphorylated Raf-1 and MEK1/2 by western blotting.

Ectopic Id-1 expression induces activation of Raf/MEK kinases. Four stable Id-1 transfectants (C2–C5) and vector control (pBabe) were examined for the expression of Id-1, phosphorylated Raf-1, MEK1/2 and total levels of Raf-1 and MEK by western blotting. Expression of actin was examined as an internal control. Quantification of specific bands was conducted by densitometric analysis and is shown as fold change at the bottom of each band as compared with pBabe. Note that increased levels of phosphorylated Raf-1 and MEK1/2 proteins are shown in the cells expressing exogenous Id-1 protein, while the total levels of these two proteins remain consistent among the cell lines studied.

**Results**

**Ectopic Id-1 expression induces activation of Raf/MEK signalling pathway in NPC**

Previously, we transfected the Id-1 gene into a NPC cell line, CNE1, which showed undetectable levels of Id-1 protein in serum-free cell culture conditions, and generated stable transfectants that expressed different levels of Id-1 protein in SFM (22). In this study, we first investigated if ectopic Id-1 expression in NPC cells had any effect on Raf/MEK signalling pathway. Using four stable Id-1 transfectant clones (CNE1-Id-1C2 to C5) and the vector control (CNE1-pBabe), we first cultured the cells in SFM for 48 h and then studied the expression of Id-1, phosphorylated Raf-1 and MEK1/2 by western blotting. As shown in Figure 1, all of the four Id-1 transfectants showed Id-1 protein expression while Id-1 was absent in the vector control. Phosphorylated Raf-1 and MEK1/2 protein levels were also increased by up to 3.8-fold in the Id-1 transfectants compared with the vector control, while the alterations of the total Raf-1 and MEK1/2 protein expression were not significant. The increased Raf-1 phosphorylation seemed to correlate with Id-1 expression levels as the clones with the highest Id-1 expression (C3, C4) also showed the highest levels of phosphorylated Raf-1 (3.8 and 2.6-fold increase, respectively). The increased phosphorylated MEK1/2 protein was also increased in all of the Id-1 transfectants. However, the phosphorylated MEK1/2 protein in C5 was much higher than that in C3, which had much higher Id-1 expression. Although the mechanism involved in this phenotype is not clear, it is possible that sporadical genetic changes occurred in C5 either during transfection or clonal expansion may have facilitated the phosphorylation of MEK1/2. Nevertheless, since activation of Raf-1 and MEK signalling is through phosphorylation (29), these results indicate that exogenous Id-1 expression in CNE1 cells has led to activation of the Raf/MEK kinase pathway.

37°C. The CNE1-pBabe and -Id-1 transfectants were maintained routinely in the selective drug puromycin (4 μg/ml), which was removed 24 h before any experiments were performed. All experiments were performed in cells cultured in serum-free medium (SFM) for at least 48 h.

3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell growth rate was measured using MTT proliferation assay kit and the procedures were described by the manufacturer (Boerhinger, St Joseph, MO). Briefly, 1000 cells were seeded in 96-well plates and then cultured in SFM for 48 h and five concentrations of Taxol (0.5–2.5 ng/ml) (Calbiochem, San Diego, CA) were added to each cell line, respectively. Cell viability was examined at 24, 48 and 72 h post-incubation time points. Before testing, 10 μl of MTT labelling reagent (5 mg/ml MTT in PBS) was added and the cells were incubated for another 4 h at 37°C. Then 100 μl of dissolving reagent (dimethylsulfoxide, DMSO) was added and the plate was incubated overnight at 37°C to dissolve the formazan crystals. The optical density (OD) was measured at a wavelength of 550 nm on a Labsystem multiskan microplate reader (Merck Eurolab, Dietikon, Schweiz). Pilot experiments were conducted to determine optimal cell concentration for comparison of the in vitro growth of cells. Each time point was performed in triplicate wells and each experiment was repeated at least three times. Results represented the OD ratio between the treated and untreated cells at indicated time points. Each data point represented the mean and standard deviation.

Western blotting

Detailed experimental procedures were described previously (27,28). Briefly, cell lysates were prepared by resuspending cell pellets in lysis buffer [20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glyceropohosphate, 1 mM NaVO4, 1 μg/ml leupeptin, 1 μg/ml aprotonin, 1 μM PMFSF]. Protein concentration was measured using DC Protein Assay kit (Bio-Rad, Hercules, CA). Equal amount of protein (30 μg) was loaded onto a SDS-polyacrylamide gel for electrophoresis and then transferred on a PVDF membrane (Amersham, Piscataway, NJ). The membrane was then incubated with primary antibodies for 1 h at room temperature against phospho-Raf (Ser259) (Cell Signaling Technology, Beverly, MA), Raf (Santa Cruz, Santa Cruz, CA), phospho-MEK1/2 (Ser217/221) (Cell Signaling Technology, MEK1/2) (Cell Signaling Technology), Bcl-2 (Santa Cruz), Bax (Santa Cruz) or actin (Roche, Mannheim, Germany), respectively. After washing with TBS-T, the membrane was incubated with secondary antibodies and the signals were visualized by ECL western blotting system (Amersham). The relative amounts of each protein were quantified by densitometry as ratios to actin and compared with pBabe, which was assigned the value of 1. Results represented three independent experiments.

Detection of apoptotic cells by propidium iodide (PI) staining

5 × 104 cells were plated in 12-well plates and 24 h later, the culture medium was changed to SFM- and MEK-specific inhibitor, PD098059 (Sigma, St Louis, MO), was added. After incubation for 48 h, taxol was added and the plate was incubated overnight at 37°C to dissolve the formazan crystals. The optical density (OD) was measured at a wavelength of 550 nm on a Labsystem multiskan microplate reader (Merck Eurolab, Dietikon, Schweiz). It has been reported that activation of the Ras/Raf/MEK pathway (22) is essential for survival against senescence in primary fibroblasts (15). Recently, we found that activation of Raf/MEK pathway was essential for Id-1-induced serum-independent growth of prostate cancer cells (16). It is possible that activation of the Raf/MEK pathway by Id-1 protein provides a protective role in cell survival against certain stresses present either intracellularly or extracellularly. Since the activation of Raf/MEK signalling pathway is found to be associated with taxol-induced cancer cell death in certain cell types (30,31), we next investigated if...
ectopic Id-1 expression in CNE1 cells could enhance Taxol-induced MAPK activation and if this activation had any protective effect on taxol induced-cell death. When we first examined the expression levels of phosphorylated MEK1/2 before and after exposure to taxol (0.5 and 2.0 ng/ml), it was found that in the untreated cells, the levels of phosphorylated MEK1/2 were higher in all of the Id-1 transfectants compared with the vector control at time zero (Figure 2, 0 h time point). In the Id-1 transfectants, after exposure to taxol, there was a further increase of up to 5.8-fold in the levels of phosphorylated MEK1/2, while the maximum increase in the vector control was 2.8-fold (Figure 2). In contrast, the total MEK1/2 levels remained unchanged in both Id-1 transfectants and the vector control. These results showed that taxol treatment of Id-1 expressing cells enhanced the phosphorylation of MEK1/2 kinase.

Since it has been suggested that in certain cell types, activation of the Raf/MEK pathway by taxol may provide a survival advantage leading to decreased cell death (32–34), we then studied if the Id-1-induced MEK1/2 activation could contribute to cellular protection against taxol-induced cell death in CNE1 cells. As shown in Figure 3, using MTT assays, we found that after exposure to five concentrations of taxol (0.5–2.0 ng/ml), the four Id-1 transfectant clones (dotted lines) showed much higher cell viability compared with the vector control and the parental cells (solid lines). Clone 2, which had the lowest Id-1 expression (Figure 1), also showed the lowest percentage of cell viability among the Id-1 transfectants, especially at later time points (48 and 72 h) after exposure to high concentrations of taxol (i.e. 2.0 ng/ml).

These results suggest that ectopic Id-1 expression has led to protection of CNE1 cells against taxol-induced cell death and that Id-1-induced Raf/MEK activation may play a role.

Inhibition of MEK1/2 phosphorylation enhances taxol-induced apoptosis in Id-1 transfectants

To further confirm the role of MEK activation in taxol-induced cell death, we treated the cells with PD098059, a specific inhibitor of MEK1/2 (35) and measured the apoptosis rate in the Id-1 transfectants and the vector control. As shown in Figure 4A, after exposure to two concentrations of PD098059 (25 and 50 μM), decreased levels of phosphorylated MEK1/2 were observed at 24 h. At 48 h post-exposure time, the levels of phosphorylated MEK1/2 were undetectable in all of the cell lines exposed to 50 μM PD098059. We then studied the effect of PD098059 treatment on taxol-induced apoptosis in these...
cells. After exposure to both PD098059 (50 μM) and taxol (2.0 ng/ml), the apoptosis rate was measured every 4 h up to 48 h after staining with PI and cells that showed the appearance of nuclear fragmentation were considered to be undergoing apoptosis (Figure 4B, arrows). As shown in Figure 4(C), in the presence of PD098059, the percentage of apoptotic cells increased in all of the cell lines with increased exposure time and the taxol-induced apoptosis rate was similar between the vector control (open columns) and the Id-1 transfectants (solid columns). These results indicate that inhibition of MEK phosphorylation demolished the protective effect of Id-1 to taxol-induced cell death in Id-1 expressing cells, further confirming the hypothesis that Id-1 induced MEK activation is essential for its protective role against taxol-induced cell death in CNE1 cells.

**Protective role of Id-1 to taxol-induced apoptosis is through regulation of Bcl-2 and Bax expression**

It has been reported that taxol induces activation of the Raf/MEK pathway, which in turn modifies the phosphorylation or expression of the regulators of the apoptosis pathway such as Bcl-2 and Bax leading to either resistance or sensitization to taxol-induced cell death (30,36,37). To further investigate if the protective effect of Id-1 in CNE1 cells was due to alterations of the Bcl-2 protein level, next we studied the expression levels of Bcl-2 after exposure to taxol in the Id-1 transfectants and the vector control. As shown in Figure 5A, we found that in pBabe cells, after exposure to two concentrations of taxol (0.5 and 2.0 ng/ml), expression of both phosphorylated (upper band) and unphosphorylated (lower band) Bcl-2 proteins was decreased (up to 90% decrease), which was associated with increased Bax protein levels in a dose- and time-dependent manner. In Id-1 transfectants, Bcl-2 protein was increased in C3 and C4, which showed the highest Id-1 expression among the four clones, while slightly decreased (up to 40% decrease) Bcl-2 was observed in clones C2 and C5. In contrast to the vector control, the Bax level was significantly decreased in all of the Id-1 transfectants. The results showed that ectopic Id-1 expression led to alterations in the ratio of Bcl-2/Bax proteins in response to taxol treatment, which may play an important part in its protection against apoptosis in CNE1 cells.

To further confirm the above observations and study the effect of blocking the MEK pathway by PD098059 on Bcl-2/Bax ratio, we then treated the cells with both MEK1/2 inhibitor PD098059 (50 μM) and taxol (2.0 ng/ml) to study if inhibition of MEK activity could lead to alterations of Bcl-2 and Bax protein levels at two time points (24 and 48 h). As shown in Figure 5B, in pBabe cells, decreased Bcl-2 and increased Bax levels were observed in a time-dependent manner, which was similar to the results found in the cells treated with taxol alone (Figure 5A). In contrast, in the Id-1 transfectants (clones C3 and C4), the addition of PD098059 resulted in decreased Bcl-2 and increased Bax protein levels compared with the cells treated with taxol alone (Figure 5A). These results demonstrated that inhibition of MEK activity resulted in decreased Bcl-2 and Bax protein ratio, which may facilitate the taxol-induced apoptosis in Id-1 transfectants. This indicates that the Raf/MEK activation is essential for the protective effect of Id-1 against taxol-induced apoptosis in CNE1 cells.

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**Fig. 4.** Inhibition of MEK1/2 results in increased taxol-induced apoptosis in Id-1 expressing cells. (A) MEK1/2 expression after exposure to its inhibitor, PD098059 (25 and 50 μM) by western blotting analysis. Note that decreased phosphorylated MEK1/2 protein is shown in all of the cell lines after exposure to PD098059 in a dose- and time-dependent manner. (B) Evidence of apoptosis in the cells treated with taxol (2.0 ng/ml) and PD098059 (50 μM) in the Id-1 expressing cells (panel 4) and the vector control (panel 3) (arrows indicate the apoptotic cells). The untreated cells were used as controls (panels 1 and 2). (C) Apoptosis rates (percentage of cells undergoing apoptosis) in the cells treated with taxol and PD098059 at different time points. Note that after exposure to both taxol and PD098059, apoptosis rates are similar in the Id-1 expressing cells compared with the vector control at tested time points. Results represent three independent experiments.
Discussion

In this study, we found that ectopic expression of Id-1 was able to protect NPC cells from taxol-induced cell death and this protective role of Id-1 was mediated through activation of the Raf/MEK pathway. Since over-expression of Id-1 is a frequent event in advanced human cancers, our results indicate a novel molecular mechanism responsible for chemodrug resistance and provide a possible treatment strategy through inactivation of Id-1 protein in certain human cancer cells.

Over-expression of downstream effectors of the MAPK signalling pathway such as EGFR and Egr-1 has been reported in NPC tumour specimens as well as cancer cell lines (23-25,38), however, the molecular mechanism involved in this process is not clear. In this study, we observed that exogenous expression of Id-1 in an NPC cell line, CNE1, resulted in activation of several kinases of the MAPK pathway and inhibition of MEK phosphorylation caused increased taxol-induced apoptosis in the Id-1 expressing cells (Figures 1-3). It is possible that Id-1 may serve as one of the upstream regulators of the MAPK pathway, which is essential for the function of Id-1 as an anti-apoptotic factor. This hypothesis is supported by our results on the increased Bcl-2 and Bax protein ratio in the taxol-treated Id-1 transfectants compared with the vector control (Figure 5), indicating that Id-1 expression in combination with activation of the MAP kinases plays an important part in protection against taxol-induced cell death in NPC cells.

One of the striking features of the undifferentiated NPC is the presence of Epstein-Barr virus (EBV) in tumour cells (39). Recently, it was reported that certain EBV-encoded proteins, such as LMP1 ad LMP2a, were also able to activate the MAP kinases both lymphoid and epithelial cells (40,41). Since CNE1 cells, as well as the majority of established NPC cell lines, are not able to maintain the EBV in vitro (26,42), therefore, activation of the MAP kinases observed in the Id-1 transfected cells in this study is unlikely to be associated with EBV.

High percentage of NPCs is sensitive to radiation, which still remains the front-line treatment option of this disease. However, combination of the radiation and chemotherapy has achieved better results, both in terms of tumour-free and overall survival (43-45). Although taxol is regarded as one of the most effective options in the treatment of NPC (43-45), resistance often develops but the molecular mechanisms remain unclear. In this study, the evidence that ectopic expression of Id-1 in NPC cells led to increased cell viability (Figure 3) indicates that over-expression of Id-1 may play a role in developing chemoresistance to taxol. Our results also suggest that up-regulation of Id-1 could be used as a marker to identify NPC cells potentially resistant to taxol.

As discussed earlier, activation of MAP kinases has been reported in a variety of human malignancies, and the expression and activity of MAP kinases are positively correlated with tumour progression (18-20). Recently, it was demonstrated...
that blockage of the MAP kinase-mediated survival signalling could lead to the enhancement of drug induced cell death (32,46,47). It was found that inhibition of MEK by its inhibitor PD098059 caused a dramatic increase in tumour cell apoptosis (32) and inhibition of the MEK pathway by the same inhibitor in combination with taxol also greatly enhanced tumour cell apoptosis (32,46,47). Therefore, it is possible that inactivation of the MAP kinase pathway may have a potential as a means of overcoming drug resistance in certain types of human cancer, although it has been reported in a sub-set of cancers that activation of MAPK pathway may facilitate taxol-induced apoptosis (36). In this study, the evidence that inactivation of MEK by PD098059 induced increased apoptosis rate (Figure 4) also supports the above hypothesis. The fact that expression of Id-1 led to activation of MAPK (Figure 1) and induced resistance to taxol via Raf/MEK pathway (Figures 2 and 3) in NPC cells provides evidence for a novel regulator of the Raf/MEK pathway and a possible novel molecular mechanism responsible for taxol resistance. Although further investigations are required to elucidate the interaction between Id-1 and the MAPK pathways, our results indicate a potential alternative way in sensitizing human cancer cells to taxol-induced apoptosis through inactivation of Id-1.

In summary, in this study, our results have suggested two important points. One is that expression of Id-1 in NPC cells provides survival advantage against chemodrug-induced apoptosis and this protective function of Id-1 is via activation of Raf/MEK signalling pathway. Secondly, our evidence indicates a novel upstream regulator of the MAPK pathways and a possible therapeutic strategy to increase drug-induced apoptosis through inactivation of Id-1 protein in NPC cells.

Acknowledgements

This work was supported by RGC grants to Y.C.W. (HKU 7314/01M and HKU7490/03M) and Area of Excellence Scheme (Project No. AoE/P-10/01), and X.H.W. (HKU7478/03 M).

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Received July 29, 2003; revised January 9, 2004; accepted January 10, 2004