Id-1 stimulates cell proliferation through activation of EGFR in ovarian cancer cells

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Increased EGFR (epidermal growth factor receptor) expression has been reported in many types of human cancer and its levels are positively associated with advanced cancers. Recently, upregulation of Id-1 (inhibitor of differentiation or DNA binding) protein was found in over 70% of ovarian cancer samples and correlated with poor survival of ovarian cancer patients. However, the molecular mechanisms responsible for the role of Id-1 in ovarian cancer are not clear. The aim of this study was to investigate the effect of Id-1 on ovarian cancer proliferation and its association with the EGFR pathway. To achieve this, we transfected an Id-1 expression vector into three ovarian cancer cell lines and examined cell proliferation rate by flow cytometry and bromodeoxyxyridine staining. We found that ectopic Id-1 expression led to increased cell proliferation demonstrated by increased BrdU incorporation rate and S-phase fraction. The Id-1-induced cell growth was associated with upregulation of EGFR at both transcriptional and protein levels. In contrast, inactivation of Id-1 through transfection of an Id-1 antisense vector resulted in downregulation of EGFR. Our results indicate that increased Id-1 in ovarian cancer cells may promote cancer cell proliferation through upregulation of EGFR. Our findings also implicate that Id-1 may be a potential target for the development of novel strategies in the treatment of ovarian cancer.

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Ovarian cancer is the second leading cause of death from gynaecologic malignancy worldwide, with a mortality rate of 114.2 per 100,000 women (Parkin et al, 2001). Approximately 75% of women present with ovarian cancer at advanced stage; therefore, prognosis for this disease is poor, with a 5-year survival rate of less than 40% (Jemal et al, 2004).

Although the molecular basis for the development of ovarian cancer is not clear, upregulation of the epidermal growth factor receptor (EGFR) is reported to be a frequent event in ovarian cancer and associated with tumour progression, invasion and poor survival rate (Kohler et al, 1989; Simpson et al, 1995; Skirnisdottir et al, 2001; Cloven et al, 2004). For example, expression of EGFR is significantly higher in malignant ovarian cancer cells compared to borderline and benign tumours (Simpson et al, 1995). A study on 226 patients with different stages of ovarian cancer found that EGFR-positive staining was evident in approximately 50% of the patients, and among them 59.8% had recurrent or died of this disease (Skirnisdottir et al, 2004). Furthermore, in a separate study, the survival time of patients (n = 111) with EGFR-positive tumour was much shorter than those patients with EGFR-negative tumours (Kohler et al, 1989). These results indicate that EGFR may be a key factor in promoting ovarian cancer growth as well as progression. Recently, using antisense technology, suppression of EGFR led to inhibition of cellular proliferation, cell adhesion and tumorigenicity in ovarian cancer cells (Alper et al, 2000).

In addition, targeting the EGFR using an active EGFR-specific tyrosine kinase inhibitor ZD1839 also resulted in the reduction of ovarian cancer cell growth (Sewell et al, 2002). These results further suggest that inactivation of EGFR pathway may provide a therapeutic target for the treatment of ovarian cancer. However, the molecular basis of the EGFR-induced ovarian cancer cell proliferation is still not clear.

Recently, Id-1 (inhibitor of differentiation or DNA binding) has been suggested as one of the upstream regulators of the EGFR pathway (Ling et al, 2004). Id-1 is a member of the helix–loop–helix (HLH) transcription factor family. It lacks the basic domain for DNA binding and acts as a dominant inhibitor of the basic HLH transcription factors by forming heterodimers (Benezra et al, 1990). Like EGFR, upregulation of Id-1 is frequently found in many types of human cancer such as breast (Lin et al, 2000), pancreas (Maruyama et al, 1999), cervical (Schindl et al, 2001), head and neck (Langlands et al, 2000) and prostate cancer (Ouyang et al, 2002a), and increased Id-1 expression levels are associated with advanced tumour stage and poor prognosis (Maruyama et al, 1999; Schindl et al, 2001; Ouyang et al, 2002a). Recently, it is reported that over 70% of ovarian cancer samples (among a total of 101 cases) are found to express Id-1 protein, while none of the nonmalignant cystadenomas shows positive Id-1 staining examined by immunohistochemistry as well as Western blotting (Schindl et al, 2003). In addition, the cancer samples with poor or moderate histological differentiation show stronger Id-1 expression than the well-differentiated tumours. Furthermore, the overall survival is much shorter in the patients with higher Id-1 expression than the patient with relatively lower Id-1 expression (Schindl et al, 2003). These results indicate that Id-1 may play an
important part not only in tumorigenesis but also progression of ovarian cancer.

The fact that both Id-1 and EGFR protein expression levels increase with progression and poor prognosis as well as chemoresistance of ovarian cancer (Alper et al., 2001; Mahle et al., 2002; Schindl et al., 2003; Cloven et al., 2004) raises a hypothesis that these two proteins may either share similar functions or regulate through same pathways. In addition, evidence from our previous studies has shown that Id-1 promotes prostate cancer cell proliferation through activation of EGFR (Ling et al., 2004), indicating that Id-1 may be an upstream regulator of EGFR. To study the role of Id-1 on ovarian cancer cell growth and its association with EGFR pathway, in this study, we first transfected an Id-1 expression vector into three ovarian cancer cell lines and then examined the effect of ectopic Id-1 expression on ovarian cancer cell proliferation using bromodeoxyuridine (BrdU) staining and flow cytometric analysis. The effect of Id-1 on EGFR expression at both transcriptional and protein levels was also determined by luciferase assay and Western blotting. These results were further verified through transfection of an antisense Id-1 vector in two ovarian cancer cell lines with high levels of Id-1. Our results suggest that ectopic Id-1 expression stimulates ovarian cancer cell proliferation and this process is mediated through upregulation of EGFR. Our results provide novel evidence to suggest Id-1 as an upstream regulator of the EGFR pathway in promoting ovarian cancer cell growth.

MATERIALS AND METHODS

Cell lines and cell culture conditions

Five ovarian cancer cell lines, Skov3 (obtained from ATCC, Manassas, VA, USA), Ovca420, Ovca432, Ovca433, Ovca429, were maintained in RPMI 1640 (Life Technologies Inc., Carlsbad, CA, USA), supplemented with 2 mM l-glutamine and 5% (v/v) foetal calf serum (FCS) at 37 °C. Ovca420, Ovca432, Ovca433 and Ovca429 were established from freshly isolated ascites or tumour explants from patients with late-stage ovarian adenocarcinomas with distinct characteristics (Rauh-Adelmann et al., 2000).

Generation of stable Id-1-expressing transfectants

The pBabe–Id-1 retroviral expression vector and its corresponding vector control were used for generation of stable transfectants. Details on the vectors as well as transfection procedures have been described previously (Ouyang et al., 2002). All the transfectants were selected and maintained in puromycin (1–2 µg ml⁻¹). The pool of more than 20 individual clones transfected with either Id-1 or pBabe was generated. Cell culture medium was changed to serum-free medium (SFM) before performing additional experiments.

BrdU staining

Detailed experimental procedures have been described previously (Wang et al., 2002a, b). Briefly, monolayer cells were grown on 4-mm Chamber slides (ICN, Biomedicals, Aurora, OH, USA) and the culture medium was changed to SFM for 48 h. Then, the cells were treated with BrdU (10 µM) for 1 h and then washed once with PBS. The cells were then fixed in cold methanol for 5 min at room temperature and washed in PBS. The cells were incubated with mouse monoclonal antibody against BrdU (1:10, Roche Diagnostics, Indianapolis, IN, USA) for 1 h at 37 °C and then with anti-mouse IgG-FITC for 1 h at 37 °C after washing with PBS. The percentage of FITC-positive cells was evaluated and at least 500 cells were evaluated in each experiment. The percentage of BrdU-positive cells in the control vector (pBabe) was considered as 100.

The error bars represent the standard deviation generated from three independent experiments.

Cell cycle analysis

Cells (5 × 10⁵) were plated in 5% FCS culture medium. After 24 h, the culture medium was replaced by SFM for 48 h. The cells were harvested by trypsinisation and then fixed in ice-cold 70% ethanol. The cells were then washed with PBS and incubated with propidium iodide (50 µg ml⁻¹) and RNase (1 µg ml⁻¹) for 30 min. Flow cytometric analysis was performed on an EPICS profile analyser and analysed using the ModFit LT2.0 software (Coulter) as described previously (Wang et al., 2002a).

Western blotting

Detailed experimental procedures were described previously (Ouyang et al., 2002b). Briefly, whole-cell lysate was prepared by resuspending cell pellet in lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (1 µg ml⁻¹ aprotinin, 1 µg ml⁻¹ leupeptin, 1 mM PMSF), and protein concentrations were measured using the protein assay kit (Bio-Rad, Hercules, CA, USA). Protein suspension from the whole-cell lysate (20 µg) was loaded onto a sodium dodecylsulphate–polyacrylamide gel (SDS–PAGE) for electrophoresis and then transferred to a PVDF membrane (Amersham, Piscataway, NJ, USA). The membrane was then incubated with primary antibody for 1 h at room temperature against Id-1, EGFR, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBS-T, the membrane was incubated with secondary antibody against mouse or rabbit IgG and the signals were visualised using ECL plus Western blotting system (Amersham, Piscataway, NJ, USA).

Luciferase assay

Cells were plated into a 12-well plate at a density of 1 × 10⁵ cells well⁻¹. After 24 h, the medium was changed to SFM. pER-1 (luciferase reporter containing the EGFR promoter, kindly provided by Dr A Johnson, NCI, MD, USA) and pRL-CMV-Luc (internal control) were cotransfected with either the pcDNA, pcDNA-Id-1 or pcDNA-Id-1-AS, respectively (Ling et al., 2004), into the cells using Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN, USA). Cells were lysed 48 h after transfection and were assayed for luciferase activity using the Dual-luciferase reporter assay system (Promega, WI, USA). Each data point represented the mean of three experiments and error bars indicated the standard deviation.

RESULTS

Generation of stable Id-1 transfectants in ovarian cancer cells

Under in vitro culture conditions, Id-1 expression is usually dependent on FCS stimulation and this characteristic is more evident in cell lines exhibiting less aggressive phenotype. For example, the androgen- or oestrogen-dependent cell lines (i.e. LNCaP (prostate cancer) and MCF-7 (breast cancer)), which represent less aggressive tumours, show serum-dependent Id-1 expression. In contrast, the androgen- or oestrogen-independent cell lines (i.e. PC3 (prostate cancer) and MDA-MB-231 (breast cancer)), which represent aggressive tumours, express the Id-1 protein constitutively regardless of serum concentrations (Lin et al., 2000; Ouyang et al., 2002b). This phenotype provides a tool for studying the direct role of Id-1 on human cancer cells through either ectopic introduction or inactivation of the Id-1 gene. In this study, we first examined Id-1 protein expression in five ovarian
cancer cell lines in a range of FCS concentrations (5, 2.5, 1 and 0%). As shown in Figure 1A, ovarian cancer cell lines Ovca420, Ovca432 and Ovca433 showed a serum-dependent Id-1 expression, while the expression of Id-1 was much less serum dependent in the Ovca429 and Skov3 cell lines. Three cell lines Ovca420, Ovca432 and Ovca433 were transfected with an Id-1 expression vector (pBabe–Id-1) or the empty control (pBabe) and stable transfectants were generated. Id-1 protein expression was examined by Western blotting after culturing in SFM for 48 h. Expression of actin was examined as an internal loading control. Note that Id-1 protein levels are much higher after ectopic expression of the pBabe–Id-1 vector in Ovca420, Ovca432 and Ovca433 cell lines. Results represent three experiments.

Figure 1 Effect of FCS on Id-1 expression in ovarian cancer cell lines and Id-1 expression in stable transfectants. (A) Western blotting analysis of Id-1 protein expression in five ovarian cancer cell lines, Ovca420, Ovca432, Ovca433, Ovca429 and Skov3, in culture medium containing different serum concentrations. Note that the expression of Id-1 is dependent on FCS in Ovca420, Ovca432 and Ovca433 cell lines but independent in Ovca429 and Skov3 cell lines. (B) Three cell lines Ovca420, Ovca432 and Ovca433 were transfected with an Id-1 expression vector (pBabe–Id-1) or the empty control (pBabe) and stable transfectants were generated. Id-1 protein expression was examined by Western blotting after culturing in SFM for 48 h. Expression of actin was examined as an internal loading control. Note that Id-1 protein levels are much higher after ectopic expression of the pBabe–Id-1 vector in Ovca420, Ovca432 and Ovca433 cell lines. Results represent three experiments.

Effect of Id-1 expression on serum-independent cell proliferation in ovarian cancer cells

To investigate the effect of Id-1 expression on ovarian cancer cell proliferation, the DNA synthesis rate and the cell cycle S-phase fraction were determined using BrdU staining and cell cycle analysis, respectively, in the cells cultured in SFM. As shown in Figure 2, after culturing in SFM for 48 and 72 h, the percentage of BrdU-positive cells was higher than the vector controls (open columns) in all three ovarian cancer cell lines, especially in Ovca420, Ovca432 and Ovca433 cell lines (Ellerbroek et al, 1998; Ahmed et al, 2002). We then transfected an Id-1 expression vector into Ovca420, Ovca432 and Ovca433 cell lines and generated stable transfectants. As shown in Figure 1B, after selection in puromycin, the Id-1 stable transfectants showed constitutively high levels of Id-1 protein expression compared to the vector controls when cultured in serum-free conditions.

Figure 2 Effect of ectopic Id-1 expression on ovarian cancer cell proliferation. (A) BrdU incorporation rate between the Id-1 transfectants and the vector controls. Cells were cultured in SFM for 48 and 72 h, respectively, and stained with an antibody against BrdU. At least 500 cells were counted in each experiment and the percentage of BrdU-positive cells was calculated and compared with the vector controls at 48 h time point, which was assigned as 1. Results presented as the mean and standard deviation from three experiments. (B) Representative results of BrdU staining. Strong nuclear FITC-positive signal was considered as BrdU positive. The background was enhanced to facilitate the counting of total cell numbers. Note that the percentage of BrdU-positive cells is higher in Id-1 transfectants than the pBabe control transfectants.
Effect of ectopic Id-1 expression on EGFR in ovarian cancer cells

As previously suggested, Id-1 promotes cell proliferation through EGFR pathway in prostate cancer cells (Ling et al, 2004). Next, we studied if the Id-1-induced cell proliferation in ovarian cancer cells was mediated through EGFR pathway. As shown in Figure 4A, the vector-transfected control Ovca420 and Ovca433 cells showed detectable basal levels of EGFR protein, while EGFR was absent in Ovca432 cells. After exogenous Id-1 expression, the EGFR protein level was increased remarkably in all of the Id-1 transfectants compared with the vector control, suggesting that ectopic Id-1 expression resulted in upregulation of EGFR protein expression.

To confirm these results, luciferase assay was performed to examine if ectopic Id-1 expression could lead to EGFR activation at transcriptional level. After cotransfection of pcDNA-Id-1 or pcDNA vectors with pER-1 (luciferase reporter containing the EGFR promoter) and pRL-CMV-Luc (internal control) was cotransfected with pcDNA or pcDNA-Id-1, respectively. Cells were lysed 48 h after transfection and assayed for luciferase activity using the Dual-luciferase reporter assay system (Promega, WI, USA). The luciferase activity of the cells transfected with pcDNA or pcDNA-Id-1 was considered as 100%. Each experiment was repeated three times and the error bars represent the standard deviation from three independent experiments. As shown in Figure 4B, 48 h after transfection, the EGFR promoter activity in Ovca420, Ovca432 and Ovca433 cells transiently transfected with pcDNA-Id-1 and the vector control pcDNA, pER-1 (luciferase reporter containing the EGFR promoter) and pRL-CMV-Luc (internal control) was cotransfected with pcDNA or pcDNA-Id-1, respectively. Cells were lysed 48 h after transfection and assayed for luciferase activity using the Dual-luciferase reporter assay system (Promega, WI, USA). The luciferase activity of the cells transfected with pER-1 and pcDNA was considered as 100%. Each experiment was repeated three times and the error bars represent the standard deviation from three independent experiments. Note that ectopic expression of Id-1 in Ovca420, Ovca432 and Ovca433 cells induces upregulation of EGFR at both transcriptional and protein levels.
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A

B

Figure 5 Effect of Id-1 inactivation on EGFR. (A) Id-1 and EGFR protein expression in ovarian cancer cells transiently transfected with an antisense Id-1 expression vector (pcDNA-Id-1-AS) and the control vector (pcDNA). Cells were cultured in SFM for 48 h after transfection and Id-1 and EGFR expression was examined by Western blotting. (B) EGFR promoter activity in ovarian cancer cells transiently transfected with Id1-AS and the vector control. PER-1 (luciferase reporter containing the EGFR promoter) and pRL-CMV-Luc (internal control) were cotransfected with pcDNA or pcDNA-Id-1-AS, respectively, to Skov3 and Ovca429 cells. Cells were lysed for luciferase assays 48 h after transfection and the luciferase activity was tested using the Dual-luciferase reporter assay system (Promega, WI, USA). Samples transfected with PER-1 and pcDNA was considered as 100%. The error bars represent standard deviation from three independent experiments. Note that inhibition of Id-1 expression in Skov3 and Ovca429 cells reduces EGFR expression at both transcriptional and protein levels.

simlar levels in these two cell lines. For example, Id-1 expression was decreased by 70% in Skov3 cells and the EGFR protein level was also reduced by approximately 70% compared to the vector-transfected cells. These results were further confirmed by luciferase assay, which showed that the EGFR promoter activity was inhibited in the cells transfected with the antisense Id-1 vector (solid columns) compared to the vector control (open columns) (Figure 5B). These results further support the hypothesis that Id-1 may be an upstream regulator of the EGFR signalling pathway.

DISCUSSION

In this study, we have demonstrated the positive effect of Id-1 expression on cell proliferation in three ovarian cancer cell lines (Figures 1–3). In addition, the evidence provided in this study that ectopic expression (or downregulation) of Id-1 led to upregulation (or downregulation) of EGFR at both transcriptional and protein levels indicates that Id-1 may be an upstream positive regulator of the EGFR signalling pathway (Figures 4 and 5). Since upregulation of EGFR is a common event in ovarian cancer, our evidence implicates a novel mechanism responsible for EGFR activation in this cancer. As EGFR is one of the key factors in promoting ovarian cancer cell growth (Alper et al, 2000, 2001; Pack et al, 2004), our results also suggest an alternative target to suppress EGFR signalling pathway through inactivation of Id-1, thus inhibiting cancer cell growth. Although the Id-1-induced cell proliferation in normal as well as in cancer cells has been reported in several previous studies (Lin et al, 2000; Ouyang et al, 2002b; Wang et al, 2002b), this study is the first to demonstrate its positive role on ovarian cancer cell growth, especially its relation with EGFR pathway in ovarian cancer cells.

Several mechanisms have been suggested for the role of Id-1 in cell proliferation. For example, Id-1 inhibits p16-induced growth arrest by leading to the bypass of replicative senescence in primary cells (Adami et al, 2001) and inactivation of Id-1 resulted in suppression of EGFR in ovarian cancer cells (Ouyang et al, 2002b). Recently, the Id-1-induced cell proliferation has been linked to the Raf-MEK and NF-κB pathways (Ohtani et al, 2001; Ling et al, 2002, 2003). Since Id-1 is a regulator of transcription, it is not surprising that it may regulate gene expression involving multiple signalling pathways. In this study, we found that ectopic Id-1 expression led to transcriptional activation of EGFR (Figure 4), while inactivation of Id-1 resulted in suppression of EGFR expression (Figure 5). Our results indicate a novel downstream effector of Id-1 in ovarian cancer cells. It is possible that increased Id-1 expression in ovarian cancer cells may provide autocrine signals to stimulate EGFR activity, resulting in promotion of cell proliferation. Since inactivation of EGFR has shown promising results in inhibition of ovarian cancer cell growth as well as suppression of metastatic phenotypes, our results suggest a new target in inhibition of EGFR signalling. Previously, it was also reported that Id-1 protected against anticancer drug taxol-induced cell death in nasopharyngeal carcinoma cells (Cheung et al, 2004) and suppression of Id-1 resulted in sensitisation to TNFα-induced apoptosis in prostate cancer cells (Ling et al, 2003). Since the front-line treatment strategy for advanced ovarian cancers is chemotherapy, downregulation of Id-1 may provide a novel strategy in improving the efficiency of chemotherapeutic drugs through suppression of Id-1-induced protection against apoptosis.

Activation of EGFR as well as upregulation of Id-1 have been associated with aggressive behaviour and poor clinical outcome in ovarian cancer patients, respectively (Schindl et al, 2003; Skirnisdottir et al, 2004). In this study, we also found that the expression of Id-1 and EGFR is not surprising that it may regulate gene expression involving multiple signalling pathways. In this study, we found that ectopic Id-1 expression led to transcriptional activation of EGFR (Figure 4), while inactivation of Id-1 resulted in suppression of EGFR expression (Figure 5). Our results indicate a novel downstream effector of Id-1 in ovarian cancer cells. It is possible that increased Id-1 expression in ovarian cancer cells may provide autocrine signals to stimulate EGFR activity, resulting in promotion of cell proliferation. Since inactivation of EGFR has shown promising results in inhibition of ovarian cancer cell growth as well as suppression of metastatic phenotypes, our results suggest a new target in inhibition of EGFR signalling. Previously, it was also reported that Id-1 protected against anticancer drug taxol-induced cell death in nasopharyngeal carcinoma cells (Cheung et al, 2004) and suppression of Id-1 resulted in sensitisation to TNFα-induced apoptosis in prostate cancer cells (Ling et al, 2003). Since the front-line treatment strategy for advanced ovarian cancers is chemotherapy, downregulation of Id-1 may provide a novel strategy in improving the efficiency of chemotherapeutic drugs through suppression of Id-1-induced protection against apoptosis.

In summary, we have provided first evidence that Id-1 plays an important part in the proliferation of ovarian cancer cells and this function is mediated through upregulation of EGFR. Although further investigations are needed to elucidate the precise molecular mechanisms responsible for the role of Id-1 in ovarian cancer, our
results suggest a novel upstream regulator of the EGFR pathway. Since inhibition of EGFR is effective in the suppression of ovarian cancer cell growth, inactivation of Id-1 may provide an alternative strategy for the treatment of this cancer.

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