Id-1 activation of PI3K/Akt/NFκB signaling pathway and its significance in promoting survival of esophageal cancer cells

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Inhibitor of differentiation or DNA binding (Id-1) is a helix-loop-helix protein that is over-expressed in many types of cancer including esophageal cancer. This study aims to investigate its effects on the phosphatidylinositol-3-kinase (PI3K)/Akt nuclear factor kappa B (NFκB) signaling pathway and the significance in promoting esophageal cancer cell apoptosis. We found elevated expression of phosphorylated forms of Akt, glycogen synthase kinase 3β and inhibitor of kappa B, as well as increased nuclear translocation of NFκB subunit p65 and NFκB DNA-binding activity, in esophageal cancer cells with stable ectopic Id-1 expression. Transient transfection of Id-1 into HEK293 cells confirmed activation of PI3K/Akt/NFκB signaling and the effects were counteracted by the PI3K inhibitor LY294002. Treatment with tumor necrosis factor-α (TNF-α) elicited a significantly weaker apoptotic response, following a marked and sustained activation of Akt and NFκB in the Id-1-over-expressing cells, compared with the vector control. The effects of Id-1 on the PI3K/Akt/NFκB signaling pathway and apoptosis were reversed in esophageal cancer cells transfected with siRNA against Id-1. In addition, inhibition of PI3K or NFκB signaling using the PI3K inhibitor LY294002 or the NFκB inhibitor Bay11-7082 increased the sensitivity of Id-1-over-expressing esophageal cancer cells to TNF-α-induced apoptosis. Our results provide the first evidence that Id-1 induces the activation of PI3K/Akt/NFκB signaling pathway, and protects esophageal cancer cells from TNF-α-induced apoptosis in vitro. Inactivation of Id-1 may provide us with a novel strategy to improve the treatment and survival of patients with esophageal cancer.

Introduction

Inhibitor of differentiation or DNA binding (Id-1) is a member of the helix-loop-helix proteins. It lacks the basic domain for DNA binding and functions as a dominant inhibitor of the basic helix-loop-helix transcription factors by forming heterodimers, thus inhibiting gene expression (1). Increased expression of Id-1 has been found in many types of human cancer including esophageal squamous cell carcinoma (ESCC) (2). Recently, Id-1 has been shown to play an important role in the regulation of cell proliferation and survival in human cancers (3,4). In addition, Id-1 promotes invasion and metastasis of human cancers (5), and the level of Id-1 protein expression is associated with poor prognosis in several types of cancers (6,7). Id-1, therefore, may play a critical role in tumorigenesis and cancer progression. The oncogenic function of Id-1 may involve multiple signaling pathways.

Materials and methods

Cell culture and drugs

Human ESCC cell line, HKESC-3, was established from a well-differentiated ESCC (25). The cell line has serum-dependent Id-1 expression. The establishment of stable Id-1-expressing clones and empty vector control clones from this cell line was described previously (11). The pooled stable transfectants, designated HKESC-3-Id-1 and HKESC-3-pBabe, respectively, and another ESCC cell line KYSE510 with high endogenous Id-1 expression even under serum-starved condition (26) were maintained in RPMI 1640 (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Gaithersburg, MD) at 37°C in 5% CO2. The HEK293 cell line, obtained from American Type Culture Collection (Rockville, MD), was maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum. TNF-α (PeproTech EC, London, UK) was diluted in culture medium to obtain the desired concentration. Unless specified otherwise, the HKESC-3-Id-1 and HKESC-3-pBabe cells were cultured in serum-free medium for 24 h before treatment or collection.

Expression vector and transient transfection

The vector containing full-length Id-1 cDNA (pDNA3-Id-1) or empty vector control (pDNA3) (27) was transiently transfected into HEK293 cells using Fugene 6 transfection reagent (Roche Diagnostics, GmbH, Mannheim, Germany).
according to the manufacturer’s protocol. Cells were collected 48 h after transfection for Western blot.

**Knock down of Id-1 expression using siRNA**

A siRNA duplex targeting Id-1 (si-Id-1) was purchased from Dharmacon (Chicago, IL) and dissolved in RNase-free distilled water. The siRNA target site is 5'-UAACGUGGCUGCUCUACGA-3', and the si-gene duplex RNA sequence used was validated previously to successfully inhibit Id-1 in a nasopharyngeal carcinoma cell line (28). The Dharmacon siCONTROL non-targeting siRNA (catalog number D-001210-02) was used as irrelevant siRNA control (si-CON). Cells were seeded into six-well plates and left for 24 h until ~50% confluent. A 5 µl aliquot of siRNA solution (20 µM) and 4 µl of Lipofectamine 2000 (Invitrogen) were each mixed with 250 µl of RPMI 1640 culture medium. The two mixtures were combined and incubated for 20 min at room temperature, then added to the cells to give a final concentration of 100 nM siRNA. The siRNA-transfected cells were used 48 h post-transfection.

**Preparation of nuclear extracts**

Cells (5 x 10^6) collected after trypsinization were washed with ice-cold phosphate-buffered saline and the pellet resuspended in 200 µl of ice-cold Buffer A (10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.9, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid, 2.5 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM dithiothreitol, 1 µg/ml NaF and 0.5 mM phenylmethylsulfonyl fluoride). After incubation on ice for 15 min, 10 µl of 1% Nonidet P-40 was added. The mixture was left at room temperature for 4 min, and then vortexed vigorously for 20 s. The cell nuclear pellet was collected by centrifugation at 3000 rpm for 3 min, washed with 100 µl of Buffer A, and collected again by centrifugation. The pellet was then resuspended in 50 µl of Buffer B (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.9, 0.4 M NaCl, 1 mM ethylenediaminetetraacetic acid, 2.5 µg/ml leupeptin, 1 µg/ml aprotinin, 1 mM dithiothreitol, 1 µg/ml NaF and 1 mM phenylmethylsulfonyl fluoride), shaken vigorously at 4°C for 15 min and then centrifuged at 14,000 r.p.m. for 15 min at room temperature. The supernatant was recovered as nuclear extract and then transferred to a fresh Eppendorf tube containing 10% v/v of glycerol. The protein concentration was assayed using Bio-Rad Dc protein assay kit (Bio-Rad, Hercules, CA) before use for Western blotting or electrophoretic mobility shift assay.

**Western blot analyses**

Preparation of whole-cell lysates and immunoblotting were described previously (11,29). The primary antibodies used include p-Akt (Ser-473), Akt, p-glycogen synthase kinase 3β (GSK3β) (Ser-9), GSK3β, p-1kB (Ser-32/36), caspase-3, cleaved caspase-3, poly (ADP-ribose) polymerase (PARP) (Cell signaling Technology, Beverly, MA), and Id-1, Id-2, Id-3, Id-4, NFκB/p65, histone H1 and actin (Santa Cruz Biotechnology, Santa Cruz, CA).

**Electrophoretic mobility shift assay**

NFκB DNA-binding activity was detected using an EMSA kit (Panomics, Redwood, CA) according to manufacturer’s instruction. Briefly, the nuclear extract was incubated in 1 x binding reaction mixture including biotin-labeled double-stranded NFκB consensus oligonucleotide for 30 min at 20°C. The mixture was separated on a non-denaturing polyacrylamide gel and then transferred to a nylon membrane (Amersham). The membrane was baked for 1 h at 80°C in a dry oven, then transferred to a UV crosslinker oven for 3 min. The shifted bands corresponding to the protein-DNA complexes were visualized relative to the unbound dsDNA. The bands were visualized after exposure to BioMax Light Film (Kodak, Rochester, NY).

**TdT-mediated dUTP nick-end labeling assay**

End labeling of exposed 3'-OH ends of DNA fragments was undertaken with the TdT-mediated dUTP nick-end labeling (TUNEL) in situ cell death detection kit Fluorescein (Roche Diagnostics) as described by the manufacturer. Briefly, the cells were fixed with 4% paraformaldehyde for 1 h, and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 min before incubating in TUNEL reaction mixture. The cells were subsequently stained with 4',6-diamidino-2-phenylindole and visualized under a fluorescence microscope with a x40 objective. Eight representative areas were randomly selected. At least 500 4',6-diamidino-2-phenylindole-positive cells were scored. The percentage of apoptotic cells was determined by dividing the number of TUNEL-positive cells by the total number of cells (4',6-diamidino-2-phenylindole-positive cells) in the corresponding area.

**Results**

**Effect of Id-1 over-expression and silencing on Akt signaling pathway**

Esophageal cancer cells with stable ectopic Id-1 expression (HKESC-3-Id-1) and the vector control cells (HKESC-3-pBabe) were used to investigate whether ectopic Id-1 expression had any effect on the Akt signaling pathway. These pooled populations of stably transfected clones were derived from parental cells that showed nearly undetectable Id-1 expression under serum-free condition (Figure 1A). Since Akt is activated through phosphorylation (30), we detected the expression of the Ser 473 phosphorylated form of Akt and its downstream target, GSK3β, in the cell lysates. As shown in Figure 1B, there was increased phosphorylation of Akt and GSK3β in HKESC-3-Id-1, although the total levels of each protein remained similar to
Akt and NF$\kappa$B (16,17), and our results above indicated that Id-1 activated both PI3K/Akt and NF$\kappa$B signaling pathways. Therefore, we hypothesized that Id-1 activates Akt through the PI3K signaling pathway. To examine whether PI3K was involved in the induction of Akt in the Id-1-over-expressing cells, esophageal cancer cells with stable ectopic Id-1 expression (i.e. HKESC-3-Id-1) were treated with a PI3K-specific inhibitor LY294002. As shown in Figure 2A, treatment with LY294002 attenuated Id-1-induced phosphorylation of Akt and GSK3B in HKESC-3-Id-1, indicating the significance of PI3K in the Id-1 activation of Akt pathway. To confirm this observation, we transiently transfected HEK293 cells with different amounts of Id-1 expression vector and studied the effects on the expression levels of Akt, GSK3B and their phosphorylated forms in the presence of LY294002. Figure 2B shows that inhibition of PI3K with LY294002 abolished the dose-dependent up-regulation of p-Akt and p-GSK3B by Id-1 seen in a parallel experiment without the addition of LY294002. These data show that PI3K is involved in the Id-1 induction of Akt activity.

**Effect of Id-1 expression on NF$\kappa$B signaling pathway**

We next investigated whether Id-1 expression had any effect on NF$\kappa$B activity. Since NF$\kappa$B translocates to the nucleus upon activation, we studied the expression of the most abundant subunit of NF$\kappa$B, p65, in the nuclear extracts and whole lysates of Id-1-over-expressing cells and the vector control cells by Western blot. As shown in Figure 3A, there was increased nuclear translocation of p65 in HKESC-3-Id-1, as indicated by increased p65 expression in the nuclear extract, compared with that of the HKESC-3-pBabe vector control cells. Since phosphorylation of IxB is required for NF$\kappa$B activation (19), we also compared the expression level of the phosphorylated form of IxB in the whole-cell lysates of the two cell lines, and found increased phosphorylated IxB in HKESC-3-Id-1 (Figure 3A). Moreover, transient transfection of HEK293 cells with increasing amounts of Id-1 expression plasmids resulted in a dose-dependent increase in nuclear p65 (Figure 3B), thus demonstrating that Id-1 was directly involved in the activation of NF$\kappa$B signaling pathway. Furthermore, electrophoretic mobility shift assay showed that the HKESC-3-Id-1 cells had higher NF$\kappa$B DNA-binding activity than the empty vector control cells (Figure 3C, lane 3 compared with lane 2). We also tested the effect of Id-1 gene silencing on NF$\kappa$B in the KYSE510 ESCC cells transiently transfected with si-Id-1 and found decreased phosphorylation of IxB and reduced nuclear translocation of p65 (Figure 3D). Taken together, these observations support that Id-1 functions as an upstream regulator of the NF$\kappa$B signaling pathway.

**Id-1-induced NF$\kappa$B activation is mediated through PI3K/Akt**

Since NF$\kappa$B is a downstream target of PI3K/Akt signaling pathway (16,17), and our results above indicated that Id-1 activated both PI3K/Akt and NF$\kappa$B, we hypothesized that PI3K/Akt signaling pathway is required for the Id-1 activation of NF$\kappa$B. To confirm this observation, we transiently transfected HEK293 cells with different doses of pcDNA3-Id-1 expression vector in the presence of LY294002. As shown in Figure 2B, the LY294002-treated cells, compared with untreated cells and vehicle treated with 50 M PI3K inhibitor LY294002 for 12 h. Western blot analysis showed reduced expression of phosphorylated forms of Akt and GSK3B in the LY294002-treated cells, compared with untreated cells and vehicle (dimethyl sulfoxide)-treated cells. HEK293 cells were transiently transfected with different doses of pcDNA3-Id-1 expression vector in the absence (lanes 1–3) or presence of LY294002 (lanes 4–6). Comparison of p-Akt and p-GSK3B expression levels in the Western blots showed that treatment with LY294002 attenuated the effect of Id-1 on Akt signaling pathway.

**Id-1 protects ESCC cells from TNF-$\alpha$-induced apoptosis through activation of PI3K/Akt/NF$\kappa$B signaling pathway**

It has been documented that the PI3K/Akt/NF$\kappa$B signaling pathway plays a role in preventing cells from undergoing apoptosis (32). Since our results indicated that Id-1-induced up-regulation of the PI3K/Akt/NF$\kappa$B signaling pathway in ESCC cells, we reasoned that activation of PI3K/Akt/NF$\kappa$B by Id-1 may protect ESCC cells against apoptosis. The parental cell line HKESC-3 was tested for sensitivity to TNF-$\alpha$-induced apoptosis using the TUNEL assay. The cells showed a dose-dependent response to increasing doses of TNF-$\alpha$ (up to 200 ng/ml) in serum-free medium (Figure 4A). We then treated HKESC-3-Id-1 (consisting of pooled stable clones of Id-1 over-expressing ESCC
cells) and HKESC-3-pBabe (vector control cells) with TNF-α at a dose of 50 ng/ml for up to 24 h. As shown in Figure 4B and C, the treatment elicited a significantly weaker apoptotic response in HKESC-3-Id-1, compared with the vector control (4.8 versus 24.5% apoptotic cells at 24 h). Western blot analysis of apoptosis-related proteins, caspase 3, PARP and their cleaved fragments in the cell lysates at different time points showed increasing levels of cleaved fragments of caspase 3 and PARP in the vector control cells, which was not apparent in the Id-1-over-expressing cells (Figure 4D). These results demonstrated that the Id-1-over-expressing cells were more resistant to TNF-α-induced apoptosis than the control cells. The time course experiment also showed a rapid and marked elevation of phosphorylated Akt and nuclear p65 in the HKESC-3-Id-1 cells, and the increase was sustained throughout the remaining duration of the treatment. In contrast, the vector control cells showed a relatively delayed and weaker stimulation of Akt phosphorylation, and only a transient increase in p65 nuclear translocation. Notably, the p65 expression level in the nuclear extract of the control cells returned to basal level at 4 h, prior to increased apoptosis indicated by elevated expression levels of cleaved fragments of caspase 3 and PARP.

If the anti-apoptotic function of Id-1 is indeed mediated through Akt/NFκB, we would expect suppression of Id-1 to reverse the effects...
seen in Figure 4. Compared with HKESC-3 (Figure 4A), the KYSE510 cell line was more resistant to TNF-α treatment (Figure 5A), which made it a good model for studying the effect of Id-1 gene silencing on apoptotic response. As shown in Figure 5B and C, knock down of Id-1 expression in KYSE510 cells transiently transfected with si-Id-1 significantly increased the sensitivity to TNF-α-induced
apoptosis. Moreover, whereas induction of Akt and NFκB activities was sustained after 12 h of TNF-α treatment in the control cells, the effect was more transient in the si-Id-1 transfected KYSE510 cells and was followed by increased expressions of cleaved caspase 3 and PARP (Figure 5D).

To provide further proof that activation of PI3K/Akt/NFκB mediates the anti-apoptotic effect of Id-1, we investigated whether suppression of PI3K/Akt/NFκB could increase the sensitivity of Id-1-over-expressing cells to TNF-α-induced apoptosis in HKESC-3-Id-1 cells (Figure 6). In the presence of TNF-α, LY294002 treatment
Several novel points arise from this study. First, we provide the first evidence that Id-1 is an upstream regulator of the PI3K/Akt pathway (Figures 1 and 2). Our finding that Id-1 activates PI3K/Akt is significant since activation of PI3K/Akt is a frequent event in many types of human cancers (13,20), but the mechanisms underlying its activation are not fully understood. Recently, it was reported that the Evi1 oncogene increases the sensitivity of colon cancer cells to taxol-mediated apoptosis through activation of PI3K/Akt (33). Whether a similar association exists between the PI3K/Akt pathway and other known oncogenes warrants further investigation.

Second, in discovering an association between Id-1 and PI3K/Akt pathway, we might have identified an important missing link in the downstream signaling of Id-1 that is responsible for some of its oncogenic functions. One of downstream target implicated in the anti-apoptotic function of Id-1 in cancer cells is NFκB, but the association has only been demonstrated in prostate cancer cells (29). Our results showing that Id-1 over-expression induced phosphorylation of IkB, nuclear translocation of p65 and NFκB DNA-binding activity in ESCC and HEK293 cells confirmed this (Figure 3), but a critical question remains as to how Id-1 activates NFκB. Since our data showed that ectopic Id-1 expression also activated the Akt pathway and protected ESCC cells from TNF-α-induced apoptosis (Figures 4 and 5), and treatment with specific PI3K and NFκB inhibitors indicated that PI3K/Akt acts upstream of NFκB (Figure 6), we believe that the PI3K/Akt pathway serves as the link between Id-1 and NFκB in promoting cancer cell survival. Previously, we reported that Id-1 up-regulates MDM2 and promotes cell proliferation in ESCC (11). Since MDM2 is a direct target of Akt (12) and is known to induce cell proliferation by directly stimulating E2F-1 (34), it is possible that Id-1 acts through PI3K/Akt and MDM2 to drive G1/S progression. In addition to growth stimulation and anti-apoptotic function, Id-1 is known to promote invasion and metastasis of breast cancer cells (35,36). Since PI3K/Akt/NFκB is documented to be involved in the regulation of cellular invasion and metastasis (12,37), it may be one of the mechanisms by which Id-1 exerts its effects on cancer progression.

Third, our findings that endogenous Id-1 expression could be inhibited by siRNA to suppress the PI3K/Akt/NFκB signaling pathway and hence increase the sensitivity of ESCC cells to TNF-α-induced apoptosis suggest a potentially novel therapeutic strategy for esophageal cancer. Although PI3K/Akt/NFκB is unlikely to be the sole mediator of Id-1-dependent cell survival, its functions in regulating cell cycle control, driving tumorigenesis and imparting chemoresistance to anticancer treatment makes it an attractive target for cancer therapy (12). A number of candidate drugs targeting this pathway, such as inhibitors of PI3K, epidermal growth factor receptor, platelet-derived growth factor receptor and mammalian target of rapamycin (mTOR), as well as monoclonal HER2 antibody, have been studied. Although the PI3K inhibitors wortmannin and LY294002 have been extensively evaluated in cultured cells as research tools, the non-selectivity of these compounds within the PI3K family and the short half-life of wortmannin or poor water solubility of LY294002 have limited their clinical use. Rapamycin, an inhibitor of the Akt downstream mTOR, also has poor aqueous solubility and chemical stability, although it has significant anti-proliferative activity in several murine tumor systems (38). A rapamycin analog, CCI-779, with improved pharmaceutical properties and comparable efficacy was approved in phase I and II of clinical studies; phase III trials are in progress (39). However, inhibitors of mTOR may not block all the functions of PI3K/Akt pathway because they only affect one of the many downstream pathways of PI3K/Akt signaling. While new reagents targeting this pathway are being developed and tested, perhaps, consideration should be given to targeting Id-1 as an alternative strategy in cancer therapy since evidence to date indicates that Id-1 has multiple effects on tumor progression including tumor growth, invasion, angiogenesis, metastasis and drug resistance. This notion is supported by a study showing that systemically targeting Id-1 expression using antisense Id-1 cDNA reduced the metastatic spread of 4T1 breast cancer cells in syngeneic BALB/c mice (5). From a therapeutic standpoint, since Id-1 is over-expressed in many types of human cancer but present at very low levels in normal tissues, this approach could have clinical relevance.

Discussion

In this study, our results indicate for the first time that Id-1 induces activation of the PI3K/Akt/NFκB signaling pathway, which may be one of the mechanisms responsible for protecting ESCC cells from TNF-α-induced apoptosis.

Fig. 6. PI3K/Akt/NFκB mediated the anti-apoptotic effect of Id-1. In the presence of TNF-α (50 ng/ml), Id-1-over-expressing cells were treated with 50 μM LY294002 (lane 4), 20 μM Bay11-7082 (lane 5) or vehicle (dimethyl sulfoxide) (lane 3) for 12 h. (A) Both inhibitors increased the sensitivity of HKESC-3-Id-1 cells to TNF-α-induced apoptosis (*P < 0.001, compared with vehicle control). (B) Western blot analyses showed that inhibition of PI3K/Akt/NFκB signaling pathway using specific inhibitors (indicated by decreased p-Akt, p-GSK3β, p-IκB and nuclear p65) led to increased expressions of apoptotic markers, cleaved caspase3 and PARP.
low levels in normal adult tissues (2), inhibition of Id-1 should have very little side effects on normal tissues. Furthermore, with the development of improved delivery systems in RNA interference technology and the recent success in application of therapeutic siRNA in non-human primates (40), RNAi-based therapeutic reagents targeting Id-1 may be a promising alternative or adjunct to cytotoxic chemotherapy for esophageal cancer.

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