Epidermal Growth Factor Receptor–Related Protein Inhibits Cell Growth and Induces Apoptosis of BxPC3 Pancreatic Cancer Cells

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Abstract
Dysregulation of the epidermal growth factor receptor (EGFR) signaling network has been frequently reported in pancreatic cancer. Inhibition of EGFR was associated with antitumor effects in both in vitro and in vivo studies of pancreatic cancer. We have previously reported the isolation and characterization of an EGFR-related protein (ERRP), which seems to be a negative regulator of EGFR. In the present investigation, we tested our hypothesis whether recombinant ERRP could be an effective inhibitor of growth of BxPC3 pancreatic cancer cells. Cell growth and apoptosis were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and apoptosis ELISA assay, respectively, in the presence and absence of recombinant ERRP in BxPC3 cells. To evaluate activation of EGFR and its downstream signaling events, levels of phospho-EGFR, phospho-AKT, and phospho-extracellular signal-regulated kinase (phospho-ERK) were determined by Western blot analysis. NF-κB activity was measured by electrophoretic mobility shift assay. Our data show, for the first time, that ERRP inhibits the growth of BxPC3 cells in a dose- and time-dependent manner. The EGFR transforming growth factor (TGf)-α–induced stimulation of cell growth and activation of EGFR was also inhibited by ERRP. These changes were accompanied by a concomitant attenuation of activation of mitogen-activated protein (MAP) kinases, AKT, and NF-κB. ERRP also induced apoptosis as evidenced by increased poly(ADP-ribose) polymerase cleavage and reduction in procaspase3. From these results, we conclude that ERRP is a potent inhibitor of growth of BxPC3 pancreatic cancer cells, which could be due to attenuation of EGFR cellular signaling processes. We also suggest that ERRP could be a potential therapeutic agent for pancreatic cancer.

Introduction
Pancreatic cancer remains one of the most difficult malignant disease to cure owing to its late stage of disease at diagnosis and its high metastatic potential. Despite its relatively low incidence, pancreatic cancer remains the fourth leading cause of cancer-related death in the United States (1), and surgery is the only curative therapy. Unfortunately, most patients with this disease are not surgical candidates and are offered chemotherapy, which is, in most cases, only palliative. Many gastrointestinal tumors, including pancreatic cancer, have been shown to overexpress the epidermal growth factor receptor (EGFR; ref. 2–4). Hence, there are many significant developments in EGFR antagonists as a potential therapeutic strategy. EGFR is a transmembrane tyrosine kinase protein. After ligand binding, EGFR undergoes homodimerization as well as heterodimerization with other members of the EGFR family. EGFR is then autophosphorylated and transphosphorylated on tyrosine residues, resulting in its association with adaptor and signaling molecules, leading to the activation of multiple intracellular signaling cascades, including phosphatidylinositol 3’-kinase–AKT and extracellular signal-regulated kinase (ERK), which ultimately leads to increased cellular proliferation and prevention of programmed cell death (3, 4). Therefore, excessive activation of EGFR-dependent pathways may have an important role in the biological aggressiveness of pancreatic cancer (5).

Multiple therapeutic strategies designed to manipulate this receptor have been developed, including specific antibodies (IMC-225, ABX-EGF; refs. 6, 7), flavonoid antioxidants (quercetin, luteolin; ref. 8), and low molecular weight EGFR-specific tyrosine kinase inhibitors (9). We recently isolated a novel negative regulator of EGFR, termed EGFR-related protein (ERRP), whose expression seems to attenuate EGFR activation (10). We have observed that recombinant ERRP inhibits the growth of colon cancer cell lines HCT-116 and Caco-2 in vitro and in vivo (11, 12). We have also found that ERRP is expressed in most benign pancreatic ductal epithelium and islet cells, but not in normal acinar cells (13). Moreover, in pancreatic ductal adenocarcinoma, the expression of ERRP frequency decreases progressively from well to moderate to poorly differentiated grade of the tumor, suggesting that a progressive loss of ERRP may partly contribute to the aggressive tumor cell growth in pancreatic adenocarcinoma (13).

In the present investigation, we tested our hypothesis whether purified recombinant ERRP could be an effective inhibitor of growth of pancreatic cancer cells in vitro. Our data show that ERRP inhibits growth of BxPC3 pancreatic cancer cell line in a dose- and time-dependent manner, with concomitant induction of apoptosis due to attenuation of EGFR and its downstream signaling. Our results suggest that ERRP could be a potential therapeutic agent for pancreatic cancer.

Materials and Methods
Cells and experimental reagents. Human pancreatic cancer cell line BxPC3 was obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in low-glucose DMEM, containing fetal bovine serum, and antibiotic solution consisting 100 units/ml penicillin sodium, 100 μg/mL streptomycin sulfate (Life Technologies, Gaithersburg, MD). Cell death ELISA kit was obtained from Roche (Indianapolis, IN).
antibodies for phosphorylated AKT, AKT, phospho-ERK, and phospho-STAT3 were purchased from Cell Signaling (Beverly, MA). Primary antibodies for phospho-EGFR (Y1173), poly(ADP-ribose) polymerase (PARP), and procaspase3 were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-EGFR antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were obtained from Pierce (Rockford, IL). Chemiluminescence detection of proteins was done with the use of a kit from Amersham Biosciences (Amersham Pharmacia Biotech, Piscataway, NJ). Protease inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all other chemicals were obtained from Sigma (St. Louis, MO).

Antibodies to epidermal growth factor receptor–related protein. Polyclonal antibodies against ERRP were generated as previously described (11). Briefly, rabbits were immunized by using an epitope from the “U” region of ERRP comprising 15 amino acids (AVTRPHLPLAQNRVS) that showed no homology with any known sequence in the database. Western blot analysis of rat liver and gastric mucosa revealed that ERRP antibodies cross-reacted strongly to a protein with molecular mass of ~55 kDa, which corresponds well with the calculated molecular mass of ERRP (11). No cross-reactivity with any member of the EGFR family of proteins (170-190 kDa) was observed, suggesting that the antibodies are specific to ERRP. Further documentation of specificity of ERRP came from the observation that antigen-neutralized antibodies to ERRP showed no immunoreactivity in benign colonic mucosa (13).

Generation of recombinant epidermal growth factor receptor–related protein. A recombinant protein was generated using the Drosophila expression system (Invitrogen, Carlsbad, CA) as described previously (11). Briefly, the expression vector pMT/V5-HisA containing the entire open reading frame of ERRP cDNA was cotransfected into (11). Briefly, the expression vector pMT/V5-HisA containing the entire expression system (Invitrogen, Carlsbad, CA) as described previously in benign colonic mucosa (13).

Results

Time course and dose response of epidermal growth factor receptor–related protein–induced growth inhibition of BxPC3 cells. To determine whether ERRP could be an effective therapeutic agent for pancreatic cancer, the effect of recombinant ERRP on cell growth of the pancreatic cancer cell line BxPC3 was examined. It is important to note that BxPC3 cells have no expression of endogenous ERRP (data not shown). We first observed that ERRP inhibited growth of BxPC3 cells in a dose-dependent manner, revealing ~50% inhibition with a dose of 2 μg/mL, and 88% inhibition with a dose of 5 μg/mL after a 72-hour treatment.
ERRP Inhibits Pancreatic Cancer Growth

Figure 1. Effect of purified recombinant ERRP on BxPC3 cell growth. A, dose response of purified recombinant ERRP on cell growth of BxPC3 cells. B, time-dependent cell growth inhibition by 5 μg/mL purified ERRP. Cells were seeded in 96-well plates at 5,000 cells per well and treated with the indicated concentrations or times of ERRP. C, treatment of ERRP with different schedules. Cells were seeded in 96-well plates at 5,000 cells per well, treated for 48 hours with either vehicle or 5 μg/mL ERRP, washed extensively, and cultured in medium devoid of ERRP or with fresh ERRP for another 48 hours. Columns, 1; control; 2; ERRP for 96 hours; 3, ERRP for 48 hours then HEPES for 48 hours; 4, ERRP for 48 hours then fresh ERRP was added for another 48 hours. After treatment, cell densities were determined by MTT assay. D, effect of ERRP on cell growth of BxPC3 cells stimulated with TGF-α (7 nmol/L) for 72 hours. BxPC3 cells were plated in standard growth medium. After 24 hours, the cells were cultured in serum-free DMEM for 48 hours. The cells were then treated with ERRP (5 μg/mL) or vehicle for 90 minutes before stimulation with TGF-α (7 nmol/L). Seventy-two hours after treatment, cell densities were determined by MTT assay. Each value represents the mean ± SD (n = 6) of three independent experiments. *P < 0.05, **P < 0.01 compared with the control.

Figure 2. Cell death assay for measuring apoptosis induced by ERRP. Cells were cultured in DMEM containing 5% fetal bovine serum and exposed to 5 μg/mL ERRP for different periods of time. Apoptosis was measured by cell death ELISA, whereas cell number was simultaneously measured by the MTT incorporation assay. Results are reported as an apoptotic index (i.e., a ratio of the total amount of apoptosis as measured by cell death ELISA per cell number equivalent as measured by MTT incorporation). Columns, mean; bars, SD. *P < 0.05, **P < 0.01 compared with the control.
Epidermal growth factor receptor–related protein reduces epidermal growth factor–induced nuclear factor-κB activity.

NF-κB plays a major role in AKT-related cell survival. To determine whether ERRP would affect the function of NF-κB, we examined the changes of NF-κB activity using EMSA. In 48-hour serum-starved BxPC3 cells, exposure of EGF (100 ng/mL) for 60 minutes caused a 200% increase in NF-κB activity (Fig. 6). This increase was further reduced by 55% by preincubation of cells with ERRP for 90 minutes (5 μg/mL; Fig. 6).

**Discussion**

EGFR signaling impacts many aspects of tumor biology, including proliferation, invasion, spreading, and apoptosis (3, 14). The activation of EGFR has been shown to enhance tumor growth, invasion, and spreading; it is also known to inhibit apoptosis.

**Figure 3.** ERRP-induced PARP cleavage and reduction in procaspase3. PARP cleavage and procaspase3 level following treatment of BxPC3 cells with 5 μg/mL ERRP for 72 to 96 hours was observed. Cells were treated with ERRP and total protein fraction was extracted, separated on SDS-PAGE, and exposed to specific antibodies using Western blotting as described in Materials and Methods. A, Western blotting results; C, a representative control group. The figures shown are representatives of three independent experiments. B, densitometric results. Films were scanned and band intensities were measured using Molecular Analyst software package. Data presented are averages of three independent experiments and are expressed as percentages of the respective controls. Columns, mean; bars, SD (n = 3).

**Figure 4.** ERRP-dependent suppression of EGFR activation. A, proliferating BxPC3 cells were plated in standard growth medium. After 24 hours, the cells were transferred into serum-free DMEM for 48 hours to permit EGFR to equilibrate to the cell surface. The cells were then treated for 90 minutes with the indicated concentrations of ERRP before stimulation with 100 ng/mL EGF. After 10 minutes, whole cell lysates were prepared and the extracts were electrophoresed and blotted for detection of total and activated (phosphorylated) EGFR. B, the histogram in each panel indicates the relative band intensity, in arbitrary densitometric units, derived from densitometric scans of three independent experiments. Results are expressed as percentage of control of phospho-EGFR/EGFR. Columns, mean; bars, SD (n = 3).
Specifically, the majority of pancreatic cancers overexpress this receptor (4, 17). Also, overexpression of EGFR in pancreatic cancer has been correlated with advanced disease at presentation and reduced median survival time (17). EGFR produces its effect on malignant cells via autocrine and paracrine loops and has been shown to bind both EGF and TGF-β.

ERRP, a 53 to 55 kDa secretory protein, has recently been isolated from the gastroduodenal mucosa (10). Our earlier studies indicated that ERRP is a negative regulator of EGFR, which inhibits proliferation and transformation of colon cancer cells in vitro by attenuating EGFR activation. In addition, our in vivo studies have shown that ERRP causes inhibition of colon cancer xenograft tumors in severe combined immunodeficient mice (11). These results indicate that ERRP could be a potential therapeutic agent for colon cancer. As EGFR also plays a predominant role in pancreatic tumor growth, it would be reasonable to assume that ERRP could be an antipancreatic cancer agent as well. The current results showing that ERRP inhibits both basal and TGF-α–induced growth of BxPC3 cells support our hypothesis.

In the previous study, we investigated the expression of ERRP in normal and neoplastic pancreas, and we found that ERRP was expressed in benign pancreatic ductal epithelium but not in ductal adenocarcinoma (13). ERRP expression decreases with decreasing tumor differentiation. Low levels of ERRP were associated with poor clinical outcome, suggesting that progressive loss of ERRP, a negative regulator of EGFR, may partly stimulate aggressive tumor cell growth in pancreatic adenocarcinoma (13). Our current results show that the growth of BxPC3 cells is inhibited by recombinant ERRP, suggesting that the restoration of ERRP function is critical for the inhibition of tumor cell growth and also induction of apoptotic processes.

We also observed that ERRP stimulates apoptotic events in BxPC3 cells after prolonged exposure (72-96 hours but not 24-48 hours), suggesting that ERRP-induced apoptosis may also partly contribute to its growth inhibitory effect. ERRP treatment for 72 hours results in increased PARP cleavage and reduction in procaspase3 levels in BxPC3 cells. The apoptosis-inducing effect of ERRP was also confirmed by apoptosis ELISA assay. These findings argue for a temporal progression wherein ERRP initially inhibits cell growth, but prolonged ERRP treatment results in an irreversible apoptotic commitment as evidenced by increased PARP cleavage and induction of apoptotic processes.

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activation. Based on these observations, it would be logical to assume that the reduction in activity of kinases downstream of EGFR is solely due to the reduction in EGFR activity. Indeed, we also found that ERRP inhibits TGF-α and EGF-induced phosphorylation of ERKs and AKT. The inhibition of EGFR, AKT, and ERK1/2 is noteworthy as these kinases play a key role in pancreatic cancer cell growth (19, 20). STAT3 has also been shown to play a role in pancreatic cancer survival (21). We found that whereas EGF and TGF-α stimulated EGFR, AKT, and ERK activation, they exert no effect on STAT3. Our results also show that although STAT3 is constitutively activated in serum-starved BxPC3 cells, this activation was not affected by ERRP. This suggests that ERRP is a specific inhibitor for EGFR pathway.

We also found that ERRP inhibited EGF-induced NF-κB activation in BxPC3 cells. This could be another mechanism by which ERRP inhibits cell growth and induces apoptosis. AKT has been shown to activate NF-κB by phosphorylation of IKK at a critical regulatory site Thr22 and subsequent degradation of IκB (22, 23). Recent report from our laboratory and others have shown that AKT is an upstream as well as a downstream target of NF-κB, because overexpression of AKT or p65 led to higher NF-κB or AKT phosphorylation, respectively (24, 25). These results indicate that there may be a cross-talk between AKT and NF-κB pathways. Nevertheless, as NF-κB is involved in cell survival, it may, in part, play some critical role in ERRP-induced growth inhibition and apoptosis of BxPC3 cells.

In summary, our current data showed that ERRP is effective in inhibiting cell growth of BxPC3 pancreatic cancer cells. The activation of EGFR, ERKs, AKT, and NF-κB was also attenuated by ERRP. Prolonged exposure of ERRP also induced apoptosis. From these results, we conclude that ERRP is a potent inhibitor of BxPC3 pancreatic cancer cell growth, which could be due to attenuation of the EGFR cellular signaling processes, suggesting that ERRP could potentially be an effective therapeutic agent for pancreatic cancer.

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References

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