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 EGF or 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). Primary
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 procaspase-3 were obtained from Upstate Biotechnology
(Lake Placid, NY). Anti-EGFR antibody was obtained from Santa Cruz Biotech-
nology (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-
ol-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 (AVTRPLHPLAQNRVS) that showed no homology
with any known sequence in the database. Western blot analysis of rat liver and gastric mucosa revealed that ERRP antibodies
reacted strongly to a protein with a 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 ERRP fusion protein was generated using the Drosophila
expression system (Invitrogen, Carlsbad, CA) as described previously
(11). Briefly, the expression vector pMTT/V5-HisA containing the entire
open reading frame of ERRP cDNA was cotransfected into Drosophila
S2 cells with a pCohaHydro plasmid, which confers hygromycin resistance.
The transfectants were selected with hygromycin at a concentration of
300 µg/mL, and individual sublines were propagated in media containing
100 µg/mL hygromycin. The stable cell lines were induced for 24 hours
with 0.5 mmol/L CuSO4 to express the ERRP fusion protein, and subsequently
lysed in lysis buffer [50 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 25 mmol/L EDTA,
1% NP40, 2.5 mmol/L Na3VO4, 0.5 mmol/L DTT, 5 mmol/L PMSF, 0.02 µmol/mL
aprotinin, and 5 µg/mL benzamidine] and incubated on ice for 15 minutes.
For every 400 µL cell suspension, 12.5 µL of 10% NP40 were added and the
cells were lysed. The disrupted cells were then centrifuged for 3 minutes
at 20,000 × g at 4°C, and the supernatant was saved as a cytosolic extract.
The pellet was resuspended in extraction buffer (22.5 mmol/L HEPES, 452
mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.1 mmol/L
DTT, 0.1 mmol/L PMSF, 0.0002 mg/mL leupeptin, 0.0002 mg/mL aprotinin,
0.05 mg/mL benzamidine). The resuspension was incubated on ice for
30 minutes with gentle shaking and centrifuged at 20,000 × g for 5 minutes.
The supernatant was stored as a nuclear extract at ~80°C. Nuclear protein
(10 µg) was subjected to electrophoretic mobility shift assay (EMSA).

Cell culture and growth assay. Human BxPC3 pancreatic cancer cells
were grown in DMEM supplemented with 5% fetal bovine serum,
100 units/mL penicillin, and 100 µg/mL streptomycin (complete medium)
at 37°C in humidified air with 5% CO2. In some experiments, cells were
cultured in serum-free medium wherever indicated. EGF (100 ng/mL) or
transforming growth factor–α (TGF–α, 7 mmol/L Invitrogen) was added to
the medium whenever necessary as indicated in the figure captions. To
perform the growth assays, cells were incubated overnight at a density
of 5,000 cells per well in 96-well plates, washed in PBS, and subsequently
in serum-free medium in the absence or presence of the specified
experimental conditions as indicated in the figure captions. After treatment,
cells were incubated with MTT (1 mg/mL) at 37°C for 2 hours and then
with isopropanol at room temperature for 1 hour. Spectrophotometric
absorbance of the samples was determined by an Ultra Multifunctional
Microplate Reader (Tecan) at 405 nm.

Electrophoretic mobility shift assay for measuring nuclear factor-
κB activity. A nonradioisotope EMSA was used for measuring NF-κB activity.
NF-κB consensus double-stranded oligonucleotide was 5’ end-labeled
with IRDye (LI-COR, Lincoln, NE) and used as a probe. The assays
were done in a final volume of 20 µL containing 20 mmol/L HEPES
(pH 7.9), 0.4 mmol/L EDTA (pH 8.0), 0.4 mmol/L DTT, 5% glycerol, 1% NP40,
60 mmol/L NaCl, 2 µg poly (dI-dC), 10 µg nuclear extract, and 2 pmol NF-κB
oligonucleotide. The samples were incubated for 30 minutes at 37°C and
were then electrophoresed through 8% polyacrylamide gel, followed by
scanning with the Odyssey Imaging System (LI-COR). Quantification of
scanned image was done using laser densitometry.

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.
Epidermal growth factor receptor–related protein induces apoptosis in BxPC3 cells. To investigate whether the growth inhibitory effects of ERRP are partially related to the induction of apoptosis, the effect of ERRP on apoptotic cell death of BxPC3 cells was examined using an ELISA-based assay, which measures cell death by quantitatively detecting cytosolic histone-associated DNA fragments. No significant apoptosis was observed after treatment with ERRP for 48 hours. However, ERRP induced apoptosis in BxPC3 cells after 72-hour treatment compared with control (Fig. 2). These data suggest that the growth inhibitory activity of ERRP is partly attributed to an increase in cell death.

Epidermal growth factor receptor–related protein induced poly(ADP-ribose) polymerase cleavage and reduction in pro-caspase3 level. The process of apoptosis is carried out by activation of a cascade of proteolytic enzymes belonging to the caspase family (16). Caspase-mediated cleavage of proteins can result in activation of PARP, which plays an important role in both DNA synthesis and repair, and it is cleaved early in the apoptotic process induced by a number of agents (16). Thus, detection of cleavage of these substrates serves as a marker for apoptosis. We examined whether ERRP treatment induces activation of caspase and degradation of PARP in BxPC3 cells.

BxPC3 cells were treated with epidermal growth factor receptor–related protein for different periods of time. In cells treated with ERRP, an appearance of a specific M₆ 85,000 cleavage product of PARP was observed after 72 or 96 hours (Fig. 3), implicating induction of PARP cleavage. No cleavage was observed in the 48-hour treatment group (data not shown) and this is
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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).

Consistent with the data presented in Fig. 2. Along with the induction of PARP cleavage by ERRP, a reduction in the level of caspase3 precursor (procaspase3) was observed in ERRP-treated cells (Fig. 3). These results provide additional evidences in support of the induction for apoptotic processes induced by ERRP in BxPC3 pancreatic cancer cells.

Epidermal growth factor receptor-related protein reduces epidermal growth factor and transforming growth factor-α–induced epidermal growth factor receptor phosphorylation. EGFR activity is required for growth of BxPC3 cells. To determine whether and to what extent ERRP affects EGFR function, BxPC3 cells were serum starved for 48 hours, and subsequently incubated with ERRP (5 μg/mL) for 90 minutes followed by exposure to 100 ng/mL EGF for 10 minutes. Fig. 4 shows a marked activation of EGFR (pEGFR) in ERRP-treated cells, whereas ERRP treatment abrogated EGF-induced activation of EGFR. The activity of EGFR was reduced by 65% in cells treated with ERRP. The basal level of phospho-EGFR was also reduced by 50% in ERRP-treated cells. Total EGFR level was not affected by ERRP treatment (Fig. 4). TGF-α–induced EGFR phosphorylation was also reduced by ERRP (data not shown).

Epidermal growth factor receptor abrogates epidermal growth factor and transforming growth factor-α–induced extracellular signal-regulated kinases and AKT activation. To determine whether and to what extent ligand-induced inhibition of EGFR activation by ERRP affects the downstream signaling events, the next experiments were done. We observed that in 48-hour serum-starved BxPC3 cells, exposure of EGF (100 ng/mL) for 10 minutes caused a marked stimulation of phosphorylation of ERKs (Fig. 5A) and AKT (Fig. 5B). These increases were totally abrogated by ERRP (5 μg/mL; Fig. 5). Neither EGF nor ERRP treatment altered the basic levels of ERK or AKT (Fig. 5). The inhibition of ERK1/2 and AKT activity is noteworthy because these kinases regulate cell growth and cell survival. As observed with EGF, TGF-α–mediated ERK activation was also abrogated by ERRP (data not shown). To test whether ERRP is a selective antagonist for ERK and AKT pathway, we also tested whether ERRP affects STAT3 activity. We found that activated STAT3 (phospho-STAT3) is constitutively expressed in 48-hour serum-starved BxPC3 cells, but the phospho-STAT3 level was not affected by treatment of BxPC3 cells with EGF treatment for 10 minutes or ERRP treatment for 100 minutes, suggesting selective effect of ERRP on ERKs and AKT pathways (Fig. 5B).

Epidermal growth factor receptor–related protein inhibits epidermal growth factor–induced nuclear factor-κB activity. NF-κB plays partial 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 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 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-α.

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. 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.

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 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.

The observation that treatment of BxPC3 cells with ERRP results in inhibition of cell growth and also attenuation of tyrosine kinases activity and tyrosine phosphorylation of EGFR suggests that ERRP exerts its growth inhibitory effect by attenuating EGFR.
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 Thr23 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. 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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