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

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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
all experiments. The 55 kDa protein was detected. Immunoaffinity-purified ERRP was used in
Drosophila related protein. ERRP fusion protein was generated using the
in benign colonic mucosa (13). 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.** 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. Western blot analysis showed that antigen-neutralized antibodies to ERRP showed no immunoreactivity in benign colonic mucosa (13).

**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 ng/mL, 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.

**Bacterial expression.** The expression of ERRP in E.coli M15 was induced using 0.5 mmol/L CuSO₄ to express the ERRP fusion protein, and subsequently cultured in serum-free medium wherever indicated. EGF (100 ng/mL) or transforming growth factor-α (TGF-α, 7 ng/mL, 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.

**Nuclear extract preparation.** Briefly, after treatment, BxPC3 cells were washed with cold PBS and collected in conical centrifuge tubes. After a 5-minute centrifugation, the pellet was resuspended in a lysis buffer (100 mmol/L HEPES, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 5 mmol/L PMSF, 0.02 mg/mL leupeptin, 0.02 mg/mL aprotinin, and 5 mg/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).

**Histone-DNA ELISA for detection of apoptosis.** The Cell Death Detection ELISA kit (Roche, Palo Alto, CA) was used for assessing apoptosis according to the manufacturer's protocol. Briefly, 8,000 cells per well were plated onto a 96-well microtiter plate in culture medium. Cells were treated with different concentrations of ERRP as well as for different periods of time. After treatment, the MITT incorporation assay was done on half of the samples to provide a measure of cell number. The other half of the samples was lysed and cell lysates were overlaid and incubated in microtiter plate modules coated with antihistone antibody. Then, samples were incubated with anti-DNA peroxidase followed by color development with ABTS substrate. The optical densities of the samples were determined using Ultra Multifunctional Microplate Reader (Tecan) at 405 nm.
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.

Treatment of epidermal growth factor receptor–related protein with different schedules. As shown in Fig. 1B, ERRP decreased BxPC3 cell growth as early as 24 hours after initiation of treatment. We also treated BxPC3 cells with ERRP with different schedules. The cells were treated with or without 5 g/mL ERRP. After a 48-hour treatment, the cells were transferred to ERRP-free medium, and cell growth was continued for another 48 hours. As shown in Fig. 1C, the removal of ERRP did not fully restore cell growth, suggesting long-lasting effect of ERRP on cell growth.

Epidermal growth factor receptor–related protein inhibits transforming growth factor-α–induced cell growth of BxPC3 cells. TGF-α, a polypeptide growth factor that binds to EGFR, is known to stimulate cell proliferation, and may also play a role in tumor growth and progression by inducing transformed phenotype (14, 15). Overexpression of TGF-α is thought to be important for tumor progression via autocrine stimulation and oncogene overexpression. Overexpression of TGF-α and EGFR has been documented in a variety of tumors (14, 15). To determine whether ERRP could inhibit TGF-α–induced cell growth of BxPC3 cells, 48-hour serum-starved cells were incubated with recombinant ERRP (5 μg/mL) in the absence or presence of 7 nmol/L TGF-α for 72 hours. TGF-α caused a 450% stimulation of cell growth of BxPC3 cells over the control after 72-hour incubation (Fig. 1D). This stimulation was completely abrogated by recombinant ERRP. ERRP itself reduced cell growth of BxPC3 cells by ~75% when compared with controls (Fig. 1D).

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 procaspase3 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 Mr 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
of the respective controls. Columns, averages of three independent experiments and are expressed as percentages measured using Molecular Analyst software package. Data presented are B figures shown are representatives of three independent experiments.

Methods.

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 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-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. Similar results were observed with other ligands of erbB family of receptors, suggesting that ERRP is a potent inhibitor of all known erbB family ligand–induced growth of cancer cells (18).

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 pro-caspase3 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

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**Figure 5.** ERRP-dependent suppression of ERKs and AKT activation. Proliferating BxPC3 cells were plated in standard growth medium. After 24 hours, the cells were transferred into serum-free DMEM for 48 hours. The cells were then treated for 90 minutes with the indicated concentrations of ERRP before stimulation with 100 ng/mL EGF. After 10 minutes of EGF stimulation, whole cell lysates were prepared and the extracts were electrophoresed and blotted for detection of total and activated ERK (A) and AKT and phospho-STAT3 (pSTAT3, B). Similar results were observed in each of three separate experiments. Phospho-STAT3 was not affected by treatments of EGF or ERRP. The histogram in each panel indicates the relative band intensity, in arbitrary units, derived from densitometric scans. Results are expressed as percentage of control of phospho-ERKs/β-actin or phospho-AKT/AKT. Columns, mean; bars, SD (n = 3).

**Figure 6.** ERRP inhibits EGF-induced NF-κB activity. A, EMSA analysis was done for BxPC3 cells. Binding of NF-κB consensus element with nuclear extracts from BxPC3 cells incubated with control, ERRP (5 μg/mL), EGF (100 ng/mL), or combination of ERRP and EGF. B, densitometric quantification of data presented in the top panel is shown in the bottom panel. The histogram indicates the relative band intensity, in arbitrary densitometric units, derived from densitometric scans of three independent experiments. Results are expressed as percentage of NF-κB activity in the control. Columns, mean; bars, SD (n = 3).
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 Thr223 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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