

EGF-Receptor Related Protein Causes Cell Cycle Arrest and Induces Apoptosis of Colon Cancer Cells *In Vitro* and *In Vivo*

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Abstract. *Background: EGF Receptor Related Protein (ERRP), a recently identified negative regulator of EGF-receptor (EGFR), has been shown to inhibit growth of colon cancer xenograft tumors in SCID mice. However, the mechanisms by which ERRP exerts its anti-tumor properties are poorly understood. The current investigation was undertaken to delineate the inhibitory mechanisms that are triggered by ERRP. Materials and Methods: For in vivo experiments, recombinant ERRP (20 µg/mouse) or an equivalent volume of vehicle was injected (away from the tumor site) every other day for 10 days to SCID mice xenotransplanted with the colon cancer cell line HCT-116. Tumor explants were obtained for further immunohistochemical analysis. For in vitro studies, the HCT-116 cell line was incubated with recombinant ERRP and apoptosis markers and cell cycle changes were evaluated. Results: Recombinant ERRP caused marked inhibition of tumor growth. This was accompanied by increased apoptosis and attenuation of ERK1/2 and Akt activities. Exposure of HCT-116 cells to recombinant ERRP for 24 hours caused apoptosis and cell cycle arrest at G₀/I-phase. Induction of apoptosis was evidenced by increased levels of cleaved caspase-3, PARP proteins and acridine orange staining. Conclusion: Our findings reveal a pro-apoptotic property of ERRP both in vitro and in vivo. We propose that ERRP functions by inhibiting the activation of the EGF-receptor signaling and its downstream effectors such as ERK and Akt kinases, underscoring the potential of ERRP for the treatment of colorectal cancer where the EGF pathway is known to be activated.*

A pathogenetic role for epidermal growth factor receptor (EGFR)-mediated signaling has been implicated in a variety of epithelial cancers including the lung, colon and head and neck (1). Targeted therapies aimed at blocking the function of this pathway have been developed and a number of them are currently being tested in clinical trials (2).

Recently, we reported the isolation and characterization of a novel negative regulator of EGF-receptor, referred to as ERRP (EGF-Receptor Related Protein; *GenBank accession # AF187818*) from the rat gastro-duodenal mucosa (3). ERRP possesses approximately 90% and 85% homology to the extracellular ligand binding domain of the rat and human EGFR, respectively (3). Although it remains to be determined whether ERRP is a splice variant of EGFR or the product of a different gene, ERRP levels are found to be high in benign human colonic and gastric mucosa and in the pancreas, but low in the respective invasive adenocarcinomas (4-6). The results of our more recent studies suggest that ERRP could be a potential therapeutic agent for colorectal cancer. This inference is based on the fact that intratumoral or subcutaneous (*away from the tumor site*) injections of recombinant ERRP cause regression of colon cancer xenograft tumors in some SCID mice and arrest tumor growth in others (6). Moreover, exposure of colon cancer cells to recombinant ERRP inhibits proliferation and attenuates basal and TGF- α -induced phosphorylation of EGFR (6).

Inhibition of the growth of tumors in response to therapeutic agents could be the result of a number of events, including stimulation of apoptosis. The present study was, therefore, undertaken to determine whether the inhibition of colon cancer xenografts in SCID mice in response to recombinant ERRP could partly be the result of induction of apoptosis.

Materials and Methods

Establishment of tumors in severe combined immunodeficient (SCID) mice. SCID mice were obtained from Taconic Farms (Germantown, NY, USA). After a period of adaptation, 2 to 3

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mice were subcutaneously injected on each flank with approximately 5×10^6 colon cancer HCT-116 cells. When tumors developed the mice were killed, the tumors were dissected, cut into small fragments and subsequently transplanted subcutaneously into similarly conditioned animals ($n=6$ for each group) by using a 12-gauge trocar. The mice were checked every other day. Once palpable tumors had developed, the mice were randomized ($n=6$ in each group) and subcutaneously injected (away from the tumor site) with either recombinant ERRP (20 $\mu\text{g/ml}$) in HEPES buffer pH 7.5 or vehicle, every other day for 10 days. The tumor length and width were measured with the aid of a caliper, and the tumor volume was calculated using the following equation: $[A \times B^2] \times 0.5$ [where A and B are tumor length and width (in mm), respectively]. At the end of the experiments the mice were necropsied and the residual tumor tissue was obtained and fixed in buffered-formalin. The formalin-fixed tissues were paraffin-embedded for immunohistochemical studies, as described below.

Antibodies utilized for immunohistochemical studies. Polyclonal antibodies to ERRP were raised in rabbits as described previously (4). Briefly, this was achieved by scanning protein data bases and identifying a region of ERRP comprising 27 amino acids (nucleotides 1580-1661), referred to as the U region, which showed no homology with any known sequence in the current data base (3). Further analysis of the U region revealed that the mid-portion of the peptide, with 15 amino acid residues, possessed the most antigenic property. A 15-mer of this region was synthesized and was used to raise antibodies in rabbits. This was carried out by Sigma-Genosys Company, (Woodlands, TX, USA).

The antibodies anti-cleaved caspase-3 (#9661), pAkt (#9277) and pMAPK (#9106) were purchased from Cell Signaling Technologies. The ERRP antibody was used at 1:1000 dilution. Caspase-3, pAkt and pMAPK antibodies were used in 1:50 dilutions.

Immunohistochemical staining. Four-micron sections of paraffin-embedded tissues were deparaffinized and microwaved for 15 min in pH:6.0 citrate buffer. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide and subsequently incubated with 5% horse serum to block non-specific binding. The slides were then incubated at 4°C for 18 h. Following incubation with primary antibodies, the avidin-biotin technique was performed with matched components (secondary biotinylated antibody and avidin-peroxidase complex) from Vector according to the manufacturer's suggested protocol. The slides were reacted with AEC (amino ethyl carbazole), counterstained with Harris' hematoxylin and examined by a pathologist blinded to sample coding.

Generation of recombinant ERRP. Recombinant ERRP was generated using the *Drosophila* expression system as described previously (6). Briefly, expression vector pMT/V5-HisA (Invitrogen) containing the entire open reading frame of ERRP cDNA was co-transfected into *Drosophila* S2 cells with pCoHygro plasmid (Invitrogen) which confers hygromycin resistance. The transfectants were selected with hygromycin at a concentration of 300 $\mu\text{g/ml}$, and individual sublines propagated in media containing 150 $\mu\text{g/ml}$ hygromycin. The stable cell lines were induced with 0.5 mM CuSO_4 to express the ERRP fusion protein. ERRP was purified from the crude cell lysate using polyhistidine antibodies conjugated to Sepharose 4B (Pharmacia) as described previously

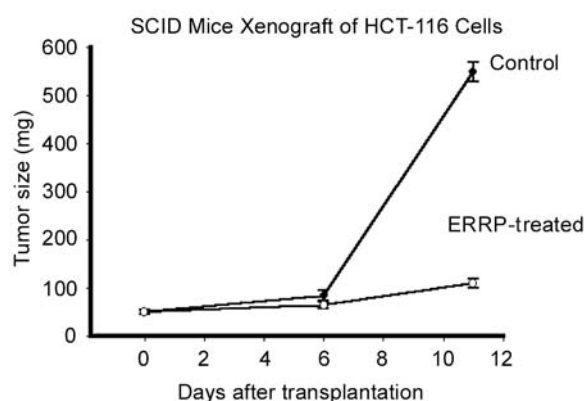


Figure 1. Tumor volume distribution in the SCID mice treated with ERRP vs. vehicle only. ERRP-treated tumors have a much smaller volume than the vehicle-treated animals; 80 ± 10.2 mg in the ERRP-treated group, vs. 550 ± 58.9 mg in the vehicle-treated group ($p < 0.05$).

(6). The bound protein was eluted with 3 M sodium thiocyanate and the fractions containing ERRP were pooled and dialyzed against 20 mM HEPES, pH 7.5 and subsequently concentrated using Amicon filter units.

Determination of ERRP-induced apoptosis in vitro. To determine whether recombinant ERRP induces apoptosis, colon cancer HCT-116 cells, maintained in DMEM/2.5% fetal bovine serum (FBS), were incubated in the absence (control) or presence of recombinant ERRP (2.5 $\mu\text{g/ml}$) for 24 h. Initial experiments to determine changes in apoptosis in response to recombinant ERRP were performed by staining the cells with acridine orange and subsequently counting the stained cells with a hemocytometer. Subsequent studies to determine changes in apoptotic events were conducted by measuring the levels of 85 kDa fragment of PARP (Poly ADP Ribose Polymerase) and procaspase-3 (both from Santa Cruz, CA, USA) by Western blot analysis as described below.

Western blot. Cells were lysed in lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 2.5 mM EDTA, 1% Triton X-100, 0.5% NP-40, 2.5mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 25 $\mu\text{g/ml}$ aprotinin and leupeptin, 50 $\mu\text{g/ml}$ Soybean trypsin inhibitor). The lysate were rotated for 30 min at 4°C and subsequently centrifuged at 11,000 rpm for 15 min at 4°C. The supernatant was used for Western blot analysis after determination of protein concentration by the protein assay kit from Bio-Rad laboratories.

Aliquots containing 50 μg of protein were separated on a 7.5% sodium dodecylsulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted to a nitrocellulose membrane. The membrane was blocked overnight with 5% nonfat dried milk in TBS-T buffer (20 mM Tris, pH 7.6, 100 mM NaCl, 0.1% Tween 20), followed by 3-h incubation with the primary antibody (1:1000 dilution) in TBS-T buffer containing 5% nonfat dried milk at room temperature. After washing three times with TBS-T buffer, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) for 1 h at room temperature. Proteins were visualized by enzyme-linked enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL,

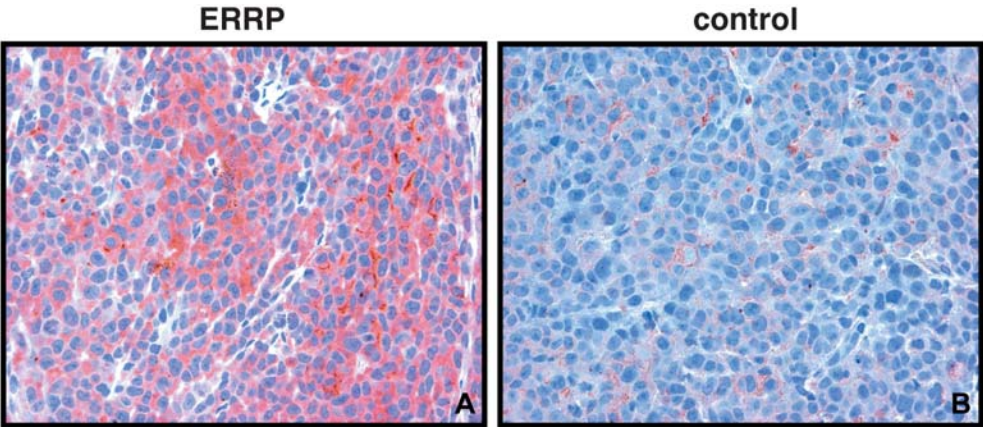


Figure 2. Immunohistochemical demonstration of ERRP protein in the xenografts obtained from mice sacrificed at the end of the experiments. The figure on the left (A) is from an ERRP-treated animal while the figure on the right (B) is from a vehicle-treated animal. (200x)

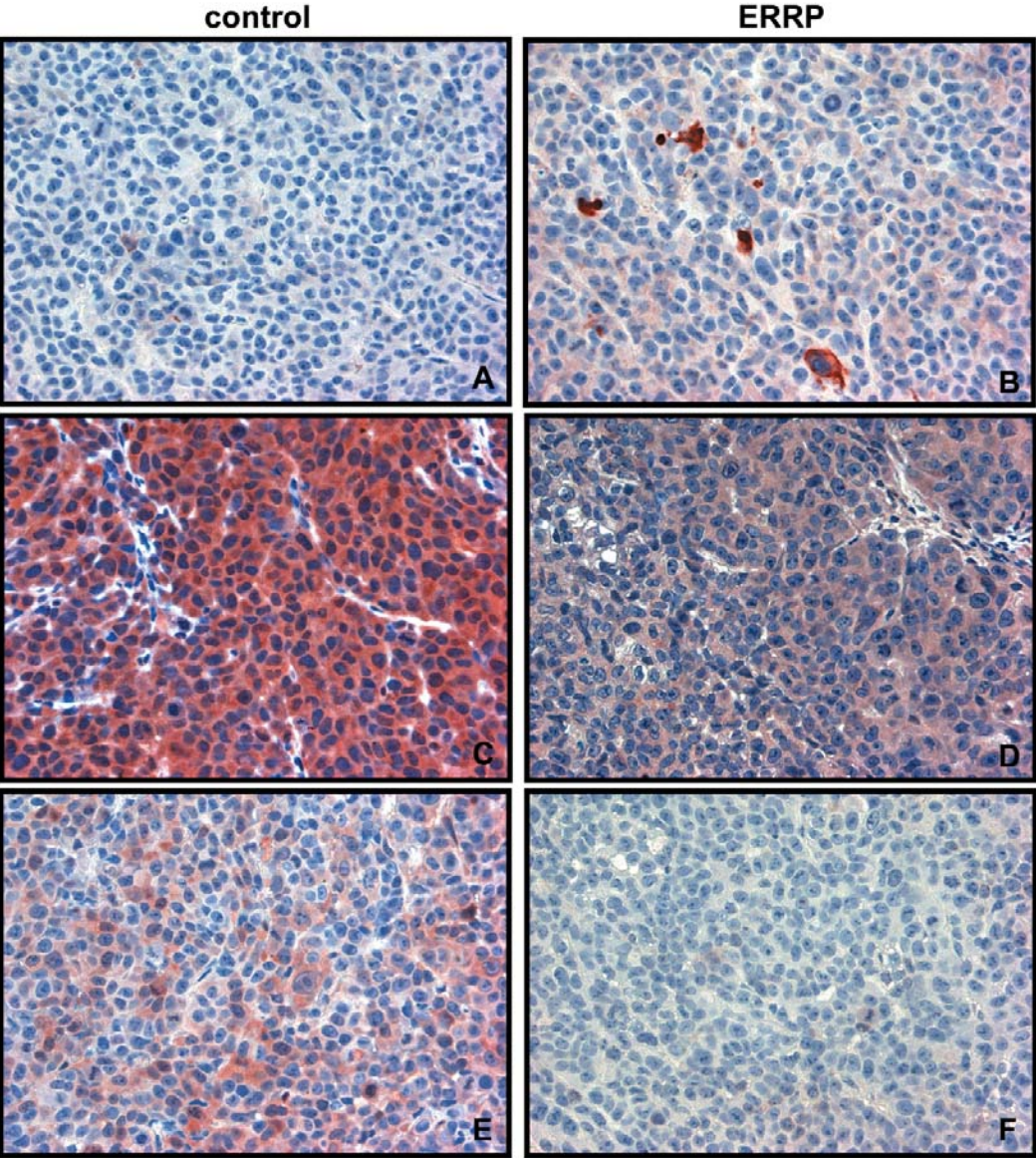


Figure 3. 3A demonstrates the cleaved-caspase-3 staining of the tumor xenograft which is treated with vehicle. 3B represents the cleaved-caspase-3 staining of the ERRP-treated tumor. 3C demonstrates the phospho-Akt staining of the tumor xenograft in the vehicle-treated animal. 3D shows the decreased expression of phospho-Akt staining in the ERRP-treated tumor. 3E shows the phospho-ERK staining of the vehicle-treated tumor. 3F shows the decreased expression of phospho-ERK in the ERRP-treated tumor. (200x)

Table I. Immunohistochemical staining scores of explanted mouse tumors.

(n=4)	Control	ERRP-treated
Cleaved caspase-3	1±0.5	6±1.2*
PMAPK	90±13	30±11*
PAkt	180 ± 10	60 ± 51*

* $p < 0.05$

USA). The membranes were stripped and probed with β -actin or α -tubulin antibodies (Boehringer Mannheim) as an internal control. Signals on the blots were visualized by autoradiography and quantitated by densitometry using the ImageQuant image analysis system (Storm Optical Scanner, Molecular Dynamics, Sunnyvale, CA, USA).

Flow cytometric analysis. The analysis of cell cycle changes in the cell lines treated with ERRP *in vitro* were determined by the simplified protocol described by Wang (7). Briefly, the cells were permeabilized with 80% ethanol and incubated in a solution containing 50 μ g/ml propidium iodide and 10 μ g/ml RNase A. A Facscalibur flow cytometer was used for data acquisition. The apoptotic component and the cell cycle phases were determined by utilizing Modfit software following acquisition of the data.

Results

In vivo studies. To study the therapeutic potential of ERRP, an efficacy trial using SCID mice with palpable tumors derived from HCT-116 cells was conducted, where recombinant ERRP (20 μ g/mouse) or vehicle (control) was administered subcutaneously (s.c.) on the back of each mouse (away from the tumor site), every other day for 10 days. As has been reported earlier (6), administration of recombinant ERRP resulted in a marked inhibition in tumor growth when compared with the vehicle-treated control (Figure 1). The average tumor volume on day 10 of the experiments was 80 ± 10.2 mg in the ERRP-treated group, vs. 550 ± 58.9 mg in the vehicle-treated group ($p < 0.05$). None of the animals treated with ERRP lost weight or showed any sign(s) of toxicity.

The animals were sacrificed at the end of the 10-day experimental period and the tumor tissues were fixed in buffered-formalin for immunohistochemical analyses. In the first set of studies, we analyzed the tumors for the relative abundance of ERRP to determine whether administered recombinant ERRP reaches the tumor. Indeed, our results revealed that, while tumors derived from mice treated with recombinant ERRP had significant expression of ERRP, no such expression was evident in tumors from the mice injected with vehicle (Figure 2).

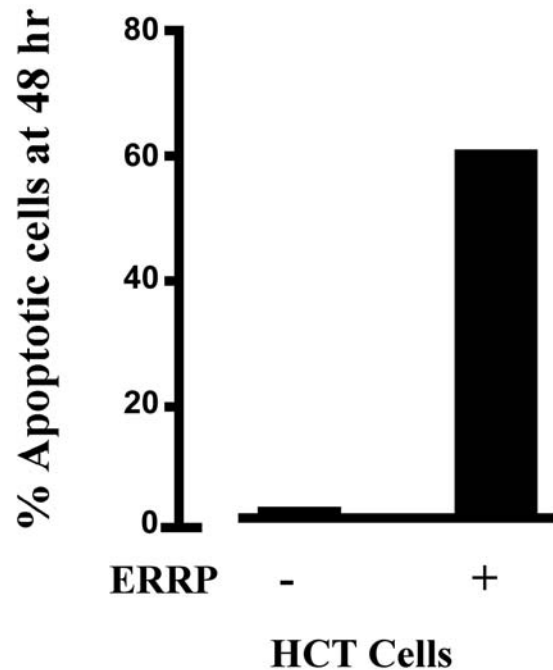


Figure 4. Graph showing the percentage of cell death determined by acridine orange staining. HCT cells were treated with vehicle vs. ERRP. Addition of ERRP increased the death rate from 5 ± 1 to 59 ± 5 ($p < 0.005$). The experiments were done in triplicate and repeated three times. A representative experiment is shown.

To determine whether ERRP-induced inhibition of tumor growth in the SCID mice xenograft of HCT-116 cells could partly be due to induction of apoptosis, we analyzed the tumors from the control and ERRP-treated mice for the expression of active caspase-3. As expected, the expression of active caspase-3 was found to be markedly higher in tumors from mice treated with recombinant ERRP, compared to those treated with vehicle (Figure 3). The nuclei staining with the antibody were counted in 10 high-power fields and an average number of apoptotic nuclei per high-power field was obtained. The mean apoptotic cell number per high-power field was 6 ± 1 in the ERRP-treated group, while the apoptotic rate in the untreated group was 1 ± 0.5 ($n = 4$; $p < 0.05$; Fisher's exact test).

We also analyzed the activation status of Akt and MAPK, which are the downstream mediators of EGFR signaling. Control tumors had high baseline pAkt and pMAPK expression, while the ERRP-treated tumors had decreased pAkt and pMAPK expression as assessed by immunohistochemistry (Figure 3). The staining intensity was scored from 0 to 3. A combined score was obtained by multiplying the intensity score with the percentage of cells staining. The combined score for pAkt decreased from 180 ± 10 to 60 ± 5 ($p < 0.05$) while the pERK staining decreased from 90 ± 13 to 30 ± 11 ($p < 0.05$), (Figure 3, Table I).

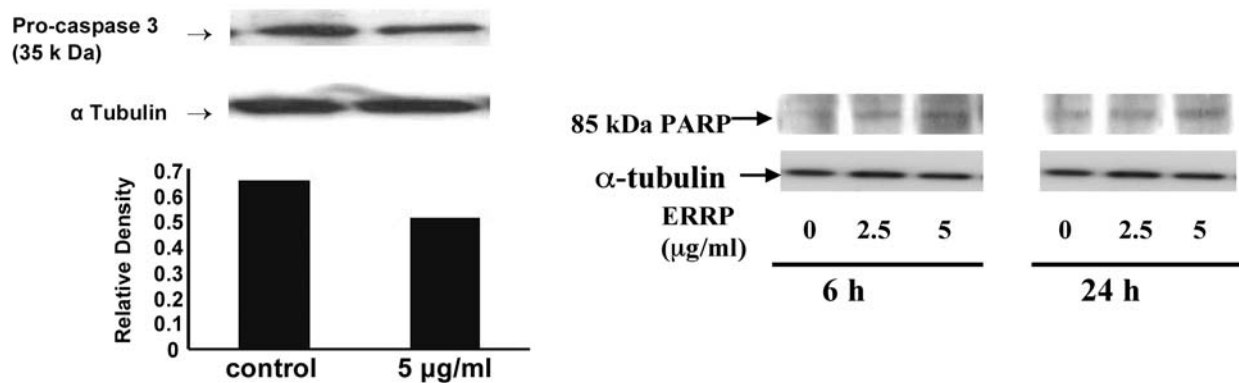


Figure 5. (A) demonstrates the pro-caspase-3 staining in the vehicle-treated cells vs ERRP-treated cells. Pro-caspase-3 levels were decreased in the ERRP-treated group suggesting increased cleavage of caspase-3. (B) shows the increase in the short form of PARP which indicates apoptotic proteolysis.

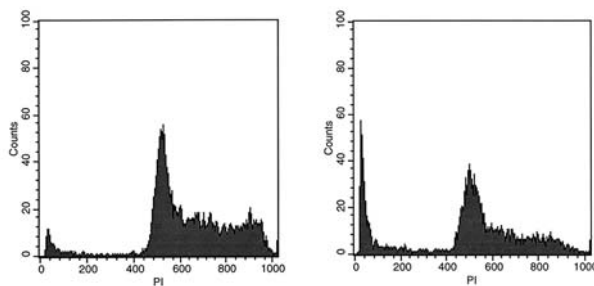


Figure 6. Cell cycle analysis by propidium iodide staining is demonstrated. The figure on the left is a cell cycle distribution curve for the vehicle-treated HCT-116 cells. The figure on the right represents the cell cycle pattern of the ERRP-treated HCT-116 cells. The sub-G₀/G₁ fraction is increased and the G₀/G₁ to G₂/M ratio is elevated in the ERRP-treated group suggesting apoptosis and cell cycle arrest at the G₀/G₁-phase.

In vitro studies. To further determine whether recombinant ERRP induces apoptosis, *in vitro* studies were performed where the colon cancer cell line HCT-116 was incubated for up to 48 h in the absence (control) or presence of recombinant ERRP. Acridine orange staining revealed a marked rise in apoptosis of the HCT-116 cells in response to recombinant ERRP. Exposure of HCT-116 cells to recombinant ERRP (5 μ g/ml) for 48 h resulted in $59 \pm 5\%$ cell death, compared to only $5 \pm 1\%$ cell death in the controls (Figure 4).

To further characterize the nature of cell death, we analyzed the expression of pro-caspase-3 and cleaved PARP expression in cell lysates obtained from the HCT-116 cells incubated with or without ERRP for 48 h. Incubation with recombinant ERRP caused a significant increase in apoptosis demonstrated by a decrease in the expression of pro-caspase-3 and an increase in the cleaved PARP expression (Figure 5).

To analyze the effects of ERRP administration on the cell cycle, we performed flow cytometric analysis following propidium iodide staining. The G₀/G₁ to G₂/M ratio increased from 1.60 ± 0.2 to 10.03 ± 0.4 in the ERRP-treated cells ($p < 0.05$). A representative cell cycle histogram is shown in Figure 6.

Discussion

Although we previously demonstrated that daily intratumoral or subcutaneous injections of recombinant ERRP inhibit colon cancer cells xenograft tumors in SCID mice, little is known about the mechanisms by which ERRP suppresses tumor growth. Our current *in vitro* data demonstrate that ERRP causes cell cycle arrest at the G₀/G₁-phase and induces apoptosis of HCT-116 cells. A similar mechanism is thought to occur *in vivo* since immunohistochemical analyses of residual tumor tissues from SCID mice following ERRP treatment show a marked increase in cleaved caspase-3 expression when compared with tumors from the controls. Moreover, the fact that the increased expression of cleaved caspase-3 in ERRP-treated tumors was associated with decreased expression of phosphorylated forms of Akt and ERKs suggests that inhibition of the Ras-Raf-MAPK and PI-3K/Akt signaling pathways may be involved in regulating this process. EGFR family members are known to activate the Ras-Raf-MAPK signaling pathway as well as the PI-3K-Akt system (8,9).

We have shown that subcutaneous injection of ERRP every other day to mice results in the accumulation of ERRP on tumor tissues. Our *in vivo* data demonstrates clearly that subcutaneous injection of ERRP is an effective way of delivering ERRP to the target sites.

EGFR receptor inhibition as a therapeutic strategy has become very popular and several clinical studies have shown the utility of inhibiting EGFR activation by antibodies or kinase

inhibitors (10). Iressa, a selective inhibitor of the EGF-receptor tyrosine kinase, which is currently in clinical use for the treatment of lung cancer, has been shown to block EGFR kinase activity and prevent activation of MAPK and Akt (11). ERRP is an endogenous peptide cloned from rat genome, that is a form of truncated EGF-receptor, but also has novel sequences that distinguishes it from the other known EGF-receptor truncated products (3). The novel sequences of ERRP endow it with inhibitory properties. Upon binding to the EGFR ligands and/or receptors, ERRP has been demonstrated to block the TGF- α -mediated activation of the EGF-receptor (6). Although it remains to be determined whether ERRP is a splice variant of EGFR or the product of a different gene, ERRP levels are found to be high in benign human colonic and gastric mucosa and in the pancreas but low in the respective invasive adenocarcinomas (4-6). Moreover, in the pancreas, the expression of ERRP is found to decrease with progression of neoplastic transformation from intraepithelial neoplasia to adenocarcinoma (4). The antibody developed against the rat ERRP has been shown to detect a 55 kD protein in human tissue protein extracts on Western blots (4,5).

Previously we have shown that expression of ERRP in the colon cancer cell lines Caco-2 and HCT-116 inhibits proliferation and EGFR activation (6). ERRP also prevents phosphorylation of EGF-receptor upon ligand binding (6).

We have previously demonstrated the co-localization of EGFR and ERRP on rat gastric epithelial cells utilizing confocal microscopy (5). Our previous studies demonstrated that ERRP is a secretory protein which functions as a regulator of EGFR activity. It is secreted to the extracellular environment and binds EGF ligands and consequently heterodimerizes with EGF receptors. Upon binding, it prevents activation of EGFR (6).

Given the fact that ligand binding and subsequent homo- or heterodimerization of EGFR are essential for activation of EGFR, any intervention that affects this process is likely to have a profound effect on EGFR signaling. Such a possibility was suggested for the 2.7 kb truncated rat liver EGFR and the 1.8 kb alternate transcript for human EGFR, whose protein product is secreted (12,13). Moreover, other studies from several laboratories have demonstrated that transfection of extracellular fragments of EGFR or kinase-negative dominant mutants of EGFR results in inhibition of EGFR phosphorylation and of anchorage-dependent or -independent growth (14-17). Wagner *et al.* (14) have demonstrated that transfection of a human EGFR cDNA fragment, generated by inserting a synthetic linker expressing only the extracellular domain of the receptor into pancreatic cancer cells (PANC-1), resulted in a marked inhibition of EGF/TGF- α induced EGFR tyrosine phosphorylation and anchorage-independent growth and increased sensitivity of cells to cisplatin. Recently, in a similar study, Matsuda *et al.* (18) demonstrated that infection of four pancreatic cancer cell lines (ASPC-1,

COLO-357, PANC-1 and T3M4) with an adenoviral vector encoding a truncated EGFR markedly attenuated EGF- and HB-EGF-dependent cell growth, tyrosine phosphorylation of EGFR family and phosphorylation of ERK, JNK and p38, as well as activating transcription factor-2. Kashles *et al.* (17) demonstrated that, although the cells transfected with wild-type EGFR and mutant EGFR lacking the cytoplasmic domain respond to EGF by forming homo- and heterodimers, only the homodimers of the wild-type EGFR underwent EGF-induced tyrosine autophosphorylation. These findings suggest that, while EGFR mutant lacking cytoplasmic domain can dimerize with wild-type EGFR in response to EGF, it does not induce the EGFR signaling process.

Since ERRP possesses 3 of the 4 ligand binding subdomains of EGFR, it is likely that ERRP exerts its action by competing with EGFR for the ligands, thereby attenuating EGFR activation. Recently, Garrett *et al.* (19) reported that a truncated EGFR lacking the extracellular domain IV of the receptor, binds EGF and TGF- α with at least a ten-fold higher affinity than the full length extracellular domain of EGFR. Since, ERRP, a naturally occurring molecule, lacks most of the extracellular domain IV, it is reasonable to predict that ERRP will also be very effective in preferentially binding/sequestering ligands of EGFR.

We believe it important to study ERRP, even though other agents have been developed to block EGFR, because ERRP may be a superior EGFR antagonist. ERRP is a widely expressed endogenous and stable secretory protein (6). Thus, the likelihood of an immune response mounted against ERRP is very small. Since ERRP is a secretory protein it could be administered systemically to inhibit tumor growth through EGFR signal inhibition. Since ERRP is down-regulated in many tumors, replacing the deficit seems theoretically more attractive than administering a different exogenous agent targeted to EGFR.

In summary, our current data demonstrate that ERRP causes cell cycle arrest and induces apoptosis in colon cancer cell lines by preventing activation of MAPK and Akt. These effects can be demonstrated both *in vitro* and *in vivo*. These studies support the potential value of ERRP as a negative regulator of EGFR signaling and demonstrate its potential as a therapeutic agent for future clinical use.

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