Dual Inhibition of Focal Adhesion Kinase (FAK) and Epidermal Growth Factor Receptor (EGFR) Pathways Cooperatively Induces Death Receptor-Mediated Apoptosis in Human Breast Cancer Cells

Vita Golubovskaya, Lucia Beviglia, Li-Hui Xu, H. Shelton Earp III, Rolf Craven, and William Cance

1 Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599.

2 Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

3 Department of Surgery, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

* To whom correspondence should be addressed: CB 7210, University of North Carolina at Chapel Hill School of Medicine, Chapel Hill, NC 27599. Phone: 919-966-5221, FAX: 919-966-8806, E-mail: cance@med.unc.edu

Running Title: Dual Inhibition of FAK and EGFR increases breast cancer cell apoptosis
SUMMARY

The focal adhesion kinase (FAK) and epidermal growth factor receptor (EGFR) are protein tyrosine kinases that are overexpressed and activated in human breast cancer. To determine the role of EGFR and FAK survival signaling in breast cancer, EGFR was stably overexpressed in BT474 breast cancer cells, and each signaling pathway was specifically targeted for inhibition. FAK and EGFR constitutively co-immunoprecipitated in BT474-EGFR-overexpressing cells. In low EGFR-expressing BT474-pcDNA3 vector control cells, inhibition of FAK by FAK-CD caused detachment and apoptosis via pathways involving activation of caspases 3 and 8, cleavage of PARP and caspase 3-dependent degradation of AKT. This apoptosis could be rescued by dominant-negative FADD (FAS-associated death domain), indicating involvement of the death receptor pathway. EGFR overexpression did not inhibit detachment induced by FAK-CD, but did suppress apoptosis, activating AKT and ERK1/2 survival pathways and inhibiting cleavage of FAK, caspases 3, 8 and PARP. Furthermore, this protective effect of EGFR signaling was reversed by EGFR kinase inhibition with AG1478. In addition, inhibition of FAK and EGFR in another breast cancer cell line BT 20, endogenously overexpressing these kinases, also induced apoptosis via the same mechanism as in the BT474-EGFR cells. The results of this study indicate that dual inhibition of FAK and EGFR signaling pathways can cooperatively enhance apoptosis in breast cancers.
INTRODUCTION

The invasion and metastasis of cancer requires a controlled process of basement membrane degradation, cellular motility, and anchorage-independent cellular survival. The process of metastasis requires a disseminating cancer cell to survive an environment that actively promotes apoptosis. Thus, for a cancer cell to effectively metastasize, it must possess survival signals that suppress apoptosis. One survival signal that recently has been shown to modulate apoptotic signaling is the focal adhesion kinase (FAK) (1-3). This non-receptor protein tyrosine kinase localizes to points of cell contact with the extracellular matrix (ECM), the focal adhesions (4,5).

FAK was originally isolated as a tyrosine phosphorylated 125 kDa protein in v-Src-transformed chicken embryo fibroblasts (6,7). FAK includes a N-terminal domain with a primary auto-phosphorylation site, tyrosine-397, that directly interacts with the Src-SH2 domain (8), a central catalytic domain with major sites of phosphorylation Tyr-576/577, and a C-terminal domain with two proline-rich segments and a focal adhesion targeting subdomain (FAT), that binds paxillin, talin, and other proteins (4,9). FAK activity is regulated by ECM receptors and integrins, and is involved in cellular processes such as spreading, motility, proliferation and survival (4,10). A non-catalytic domain of FAK, FRNK (p41/p43) is expressed in chicken embryo cells (11), initiated from an alternative promoter and start site residing within an intron (12). Ectopic expression of FRNK caused dephosphorylation of FAK at Tyr-397 (13) and blocked FAK-mediated fibroblast migration (14).
FAK was shown to be overexpressed compared to normal tissue counterparts in many human tumors, including breast, colon, and thyroid carcinomas (15-18). In human tumor cells, inhibition of FAK expression with antisense oligonucleotides to FAK or overexpression of the FAT domain led to cell rounding, detachment, reduction of invasion, and apoptosis (1);(19);(20);(20);(21);(22). Furthermore, FAK has been shown to suppress both transformation-associated apoptosis (2) as well as anoikis (detachment induced apoptosis) of epithelial cells (23), suggesting that one function of FAK is to promote survival in cells subjected to apoptotic signals. Consistent with this hypothesis, constitutively active forms of FAK prevented anoikis and stimulated transformation of epithelial cells, resulting in anchorage-independent growth and tumor formation in nude mice (23). Further evidence for the anti-apoptotic role of FAK was shown in the leukemic cell line, HL-60, where FAK was associated with activation of NF-κB and inhibition of caspase-3 (24). Conversely, caspases-3 and 6 may promote apoptosis, in part, by cleaving FAK and generating a C–terminal FRNK-like polypeptide (25).

Recently, FAK was shown to be associated with the epidermal growth factor receptor (EGFR), also known as ErbB-1 (26); (27). When epidermal growth factor (EGF) binds to the 170kDa EGFR, receptor homo- and hetero-dimerization is promoted, activating receptor tyrosine kinase activity (28) and downstream signaling [reviewed in (29-31),(32)]. The EGFR is overexpressed or activated by autocrine growth factors in many types of tumors, including breast (33,34), thyroid, (35), ovarian (36), colon (37), head and neck (38), and brain (38);(39). Furthermore, EGFR overexpression has been linked to a poor prognosis in breast cancer (40); (32), and may promote proliferation, migration, invasion, and cell survival, as well as inhibition of apoptosis (41) (42) (43). Recent reports have suggested that
FAK serves to integrate EGFR signals upon EGF induction, promoting tumor cell motility and invasion (27), (22). However, another report suggests that FAK and EGFR are constitutively associated (26). Thus, the relationship between EGFR signaling and FAK expression and activity during progression from noninvasive to an invasive and metastatic tumor phenotype is unknown, nor has their cooperation in preventing apoptosis been mechanistically examined.

In the present study, we examined the role of FAK and EGFR in survival signaling in a human breast cancer cell line model system of EGFR overexpression. We stably overexpressed EGFR in a cell line that endogenously overexpresses FAK (BT474-EGFR cells) to compare the effects of EGFR survival signaling with the parental cell line without EGFR (BT474-pcDNA3 cells). We have demonstrated that dual inhibition of FAK and EGFR cooperatively caused apoptosis in breast cancer cells. In breast cancer cells that stably overexpressed EGFR, there was a constitutive association between FAK and EGFR. Furthermore, EGFR signaling suppressed death receptor-mediated apoptosis induced by FAK-CD. The mechanism included activation of AKT and ERK signaling pathways as well as protection of FAK from caspase degradation that was reversed by EGFR kinase inhibitor. Dual inhibition of FAK by FAK-CD and EGFR by AG1478 cooperatively enhanced apoptosis in human breast cancer cell lines, via inhibition of signaling that involved both TNF family receptor-dependent AKT and ERK 1/2 pathways. This is the first report of the role of FAK and EGFR in apoptosis that shows that simultaneous inhibition of FAK and EGFR can be critical in induction of apoptosis in breast cancer cell lines.
EXPERIMENTAL PROCEDURES.

Cells and cell culture. BT474 breast carcinoma cells, described by Xu et al.,(2) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 5 µg/ml insulin and 1 µg/ml penicillin/streptomycin. The clone of BT474 cell line used in this study had low expression of EGFR (Her-1) and Her-2. BT20 breast carcinoma cells, overexpressing EGFR (44) were maintained in Essential modified Eagle’s medium containing 10% FBS. Cell lines were incubated at 37°C in 5% CO₂ humidified incubator. For EGF stimulation (Western blotting, immunoprecipitation), cells were serum starved overnight in serum-free medium. In FAK-CD induced apoptosis experiments, EGF was added for 3 times at 0, 6 and 20 or 22 hours of adenoviral infection. EGF (Calbiochem) was used at dose 10 ng/ml for 10 min for EGF-dependent signaling experiments. For inhibition of EGF stimulation, pretreatment with EGFR kinase inhibitor, AG1478 was done at dose 5 µM for 15 min. In FAK-CD induced apoptosis experiments, BT474 cells were incubated with 5 µM AG1478 for 24 hours and BT20 cells were incubated with AG1478 for more than 72 hours at 5 µM dose (added every 24 hours fresh).

Antibodies and reagents. Polyclonal antibodies to pp125 FAK (A17) were obtained from Santa Cruz Biotechnology and anti-phospho-ERK1/2 antibodies were obtained from Promega Life Science. Monoclonal anti-FAK (4.47) antibody and polyclonal anti-ERK1/2 were from Upstate Biotechnology, Inc. Phospho-Ser-AKT and total AKT antibodies were from Cell Signaling Technology Inc. Monoclonal EGFR antibody, HRP-RC20 antibody for phospho-tyrosine, monoclonal caspase-3 and PARP antibodies were ordered from Transduction Labs. Monoclonal anti-caspase-8 antibody was from PharMingen. Phospho-FAK(pY397) and Src(pY418) antibodies were from Biosource International, Inc. Vinculin,
α-Tubulin, β-actin antibodies were obtained from Sigma. Monoclonal anti-HA antibody was from Roche Molecular Biochemical. Tyrphostin, inhibitor of EGFR autophosphorylation, AG1478 was obtained from Calbiochem. Inhibitor of ERK1/2, PD98059 was obtained from Upstate Biotechnology Inc. Caspase inhibitors were obtained from Calbiochem.

Adenoviral Infection. Recombinant adenoviruses carrying LacZ gene, Ad-LacZ, and HA tagged FAK-CD gene, coding 693-1052 amino acids of FAK, Ad-FAK-CD and dominant-negative FADD (Ad-ΔFADD) were propagated by Dr. J. Samulski and the Gene Therapy Center Virus Vector Core Facility of the University of North Carolina and described in (2). Cells were plated at 1.5x10^6 in 100-mm culture plates and after 24 hours of attachment were infected with adenoviruses at optimal concentration in a 7 ml medium with 10% serum. Optimal viral concentration was determined by infection of cells with different viral concentrations and viral titer that produced greater than 95% cell infectivity was used. The optimal viral titer was 500 ffu (focus forming units)/cell, obtained from the Gene Therapy Center Virus Vector Core facility that produced 99% cell infectivity and no toxic effects for Ad-LacZ transduction, checked by X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) staining. The same viral titer was used for Ad-(HA-tagged) FAK-CD infection, causing >90% of cell infectivity, determined by HA-immunostaining. For co-infection experiments, ΔFADD adenovirus was used with FAK-CD at concentration of 333 viral particles/cell that was shown to produce high levels of FADD protein inside BT474 cells (2). For BT474 cells, caspase inhibitors (Ac-DEVD-CHO, caspase-3 inhibitor) and (Ac-YVAD-CHO, interleukin-converting enzyme inhibitor) were added at concentration 50 µM (2) for 1 hour before adenoviral infection and were present during incubation with adenoviruses for 23-24
hours. For BT 20 cells, cell-permeable caspase-3 inhibitor (DEVD-CHO) (Calbiochem) was added at concentration 5 µM for 1 hour before infection was and present during adenoviral incubation, added fresh every 24 hour. PD98059, ERK1/2 inhibitor was used at 10 µM concentration, added for 15 minutes before adenoviral infection and was present during incubation with adenoviruses for 24 hours. In these conditions, PD98059 inhibited phosphorylation of ERK1/2 in Ad-FAK-CD-infected BT474-EGFR cells (not shown).

**Staining with X-gal.** Cells were infected with Ad-LacZ, as described above. Briefly, 24 hours after infection cells were fixed for 10 min on ice with fixing solution: 2% formaldehyde, 0.2% glutaraldehyde in 1xPBS. After washing in 1xPBS for 2 times, cells were stained for 1-3 hours with X-gal staining solution, 1 µg X-gal/ml in the X-gal buffer: 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 2 mM MgCl₂ in 1xPBS. X-gal positive (blue) cells were counted for determining of infection efficiency.

**Transfections.** To make a stable BT474 cell line expressing EGFR, cells were transfected with 10µg of pcDNA3 plasmid alone to create BT474-pcDNA3 vector control cells or with 10µg EGFR-pcDNA3 plasmid (kindly provided by Dr. David Lee) to create the BT474-EGFR cells. Transfections were accomplished with 20 µl of Lipofectamine (Gibco,BRL) in a 100 mm dish, according to the manufacturer’s protocol. Stable BT474-EGFR and BT474-pcDNA3 clones were obtained using RPMI medium with 500 µM of selective antibiotic, Geneticin, (G418) (Gibco, BRL). Expression of EGFR was checked by Western blotting with anti-EGFR antibody and a clone with maximal EGFR expression was used for the study.

**Immunoprecipitation and Western blotting.** Cells were washed twice with cold 1xPBS and lysed on ice for a 30 min in a buffer containing: 50 mM Tris-HCl (pH 7.5), 150 mM
NaCl, 1% Tryton-X, 0.5%NaDOC, 0.1% SDS, 5mM EDTA 50 mM NaF, 1mM NaVO3, 10% glycerol and protease inhibitors: 10 µg/ml leupeptin, 10 µg/ml PMSF, 1 µg/ml aprotinin. The lysates were cleared by centrifugation at 10 000 rpm for 30 min at 4°C. Protein concentration was determined using BioRad Kit. The cleared lysates with equivalent amount of protein were incubated with 5 µl of primary antibody for 1 hour at 4°C and 25 µl of protein A/G agarose beads (Oncogene Research Products Inc). The precipitates were washed with the lysis buffer 3 times and resuspended in 30 µl of 2 x Laemmlli buffer. For Western blotting, boiled samples were loaded on Ready SDS-10% PAGE gels (Bio Rad, Inc). Phosphorylation status of examined proteins was detected with horseradish peroxidase-linked anti-phosphotyrosine antibody, HRP-RC20 (Transduction Labs) in 1% BSA-TBS-T buffer. The blots were stripped in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 100 mM β-mercaptoethanol at 60°C for 30 min and reprobed with primary antibody for checking equal loading of proteins. Immunoblots were developed with chemiluminescence Renaissance reagent (NEN Life Science Products, Inc).

**Immunostaining.** Attached or detached suspended cells (collected by centrifugation and spread evenly on the slide) were fixed in 4% paraformaldehyde in 1xPBS for 10 min and permeabilized with 0.2% Tryton-X 100 for 5 min on ice. Cells were blocked with 25% normal goat serum in 1xPBS for 30 min, washed in 1xPBS and incubated with primary antibody diluted 1:200 in 25% goat serum in 1xPBS. Cells were washed in 1xPBS for 3 times and a secondary Rhodamine (TRITC)-conjugated antibody (1:400 dilution in 25% goat serum) was applied to the cover slip. After 3 times washing with 1xPBS, cells were incubated with FITC-BodipyFL-phallacidin for actin staining (1:25 dilution in 25% goat serum) (Molecular Probes, Inc.). For co-immunostaining experiment, cells were incubated
with another primary antibody diluted 1:100 in 25% goat serum in 1xPBS for 1 hour. After washing in 1xPBS for 3 times, a secondary FITC-conjugated antibody (1:100 dilution) was applied to the cover slip.

**Apoptosis assay.** Detached cells were collected by centrifugation, fixed in 3.7% formaldehyde in 1xPBS for 10 min, stained with Hoechst 33342 or spread evenly on a slide for TUNEL staining. In brief, Hoechst 33342 in 1xPBS solution (1 µg/ml) was added to the fixed cells for 10 min, cells were washed with 1xPBS for two times and spread evenly on the slide. TUNEL assay was done with ApopTag Fluorescein In Situ Apoptosis Detection kit (Intergen, NY) according to the manufacturer’s protocol. Simultaneous staining and quantification of apoptotic cells with TUNEL assay and Hoechst methods produced very similar results. The percent of apoptotic cells was calculated as a ratio of apoptotic cells / total number of cells in three independent experiments in several fields with the fluorescent microscope.

**RESULTS**

**BT474-EGFR cells: EGF-dependent tyrosine phosphorylation and its inhibition by AG1478.**

To test the relationship between FAK and EGFR in breast cancer cell survival, we created a model system of EGFR overexpression in a clone of the BT474 breast carcinoma cell line (2), a cell line that expresses high levels of p125FAK, but minimal levels of EGFR. Parental BT474 cells were stably transfected with the EGFR-pcDNA3 plasmid. These cells (called BT474-EGFR cells) expressed high levels of EGFR compared to the pcDNA3 vector control cells (called BT474-pcDNA3 cells) (Fig. 1A). In BT474-EGFR cells, treatment with
EGF at a dose of 10 ng/ml rapidly increased EGFR tyrosine phosphorylation, and this effect was inhibited in the presence of the tyrphostin AG1478, an EGFR kinase inhibitor (Fig. 1B). The BT474-EGFR cells had a higher level of tyrosine phosphorylated cellular proteins than the vector control cells (Fig. 1C), and both BT474-pcDNA3 and BT474-EGFR cells rapidly increased phosphorylation of ERK1/2 at 10 min upon EGF stimulation and reversed this effect by AG1478 treatment (Fig. 1D). In addition, AKT (Ser-473) and FAK (pY397 and pY577) (not shown) were highly phosphorylated in BT474-EGFR cells.

**FAK and EGFR constitutively associate in BT474-EGFR cells.**

To test whether FAK and EGFR were associated in the BT474-EGFR cells, EGFR and FAK were immunoprecipitated using anti-FAK monoclonal antibody (Fig. 2 A, left panel). In these experiments, anti-FAK antibodies precipitated EGFR that was tyrosine phosphorylated in the presence of EGF (Fig. 2 A, left top panel). Immunoprecipitation with anti-EGFR antibodies also precipitated FAK (Fig. 2 A, right panel). The FAK and EGFR association was constitutive, as detected in the presence and absence of EGF (Fig. 2 A). Thus, FAK and EGFR are physically associated in these breast cancer cells. Tyrosine phosphorylation of total cellular FAK was not affected by EGFR inhibitor, AG1478 (Fig. 2 A, left panel), although a small portion of phosphorylated FAK that is associated with EGFR is inhibited by AG1478 (Fig. 2 A, right top panel). Dual immunofluorescence assays in individual cells demonstrated that FAK and EGFR were co-localized at focal adhesions in BT474-EGFR cells (Fig. 2B, left panel) and also in BT20 cells, endogenously overexpressing EGFR (Fig. 2B, right panel). These results show that FAK and EGFR associate and colocalize in the focal adhesions of these breast cancer cells.
Overexpression of EGFR suppresses apoptosis induced by FAK downregulation.

Next, we tested whether overexpression of EGFR protected breast cancer cells from detachment and apoptosis induced by FAK inhibition. In these experiments, we downregulated FAK function using Ad-FAK-CD in the BT474-EGFR cells as well as the BT474-pcDNA3 vector control cells. To inhibit FAK, we used an adenoviral construct of the carboxy-terminal domain (Ad-FAK-CD) that has been shown to act as a dominant negative for FAK function (2,45,46). In these experiments, we used Ad-Lac Z as a control at equal multiplicity of infection (MOI). These conditions resulted in greater than 95% infectivity as assessed by X-gal staining for Ad-LacZ (Fig. 3A) and by HA-immunostaining for HA-epitope-tagged Ad-FAK-CD (Fig. 3B).

After 24 hours of FAK-CD expression, there were similar levels of loss of adhesion in the vector control cells (93±6 %) as well as the EGFR-overexpressing cells (83 ±17 %) (Fig. 4A) and only 0.5% detached by control Ad-LacZ at the same dose (not shown). Furthermore, inhibition of EGFR kinase activity with AG1478 did not enhance the loss of adhesion (Fig. 4A). These results shows that overexpression of EGFR does not augment the ability of BT474 cells to resist loss of adhesion induced by the inhibition of FAK function.

In contrast, EGFR suppressed apoptosis induced by FAK inhibition with Ad-FAK-CD. After 24 hours of Ad-FAK-CD infection, detached BT474-EGFR cells had significantly reduced levels of apoptosis (66%), compared to BT 474-pcDNA3 cells (97%) (Fig. 4B). Treatment of the BT474-EGFR cells with EGF (10 ng/ml) did not enhance this resistance to apoptosis, indicating that the maximal effect was already obtained by the background autophosphorylation and signaling caused by EGFR overexpression. However, inhibition of EGFR signaling with AG1478 in the BT474-EGFR cells increased the level of apoptosis to...
that of control BT474-pcDNA3 cells (Fig. 4B). In Hoechst-stained cells (Fig. 4C), apoptotic condensed nuclei with fragmented chromatin in BT474-EGFR cells were maximal with both FAK and EGFR inhibition. In addition, when FAK was inhibited by FAK-CD and ERK1/2 was inhibited by the PD98059 (ERK1/2 inhibitor) in the BT474-EGFR cells, the levels of apoptosis were increased to that of BT474-pcDNA3 vector controls (Fig. 4D), suggesting that ERK1/2 enhances survival signaling in these breast cancer cells. Taken together, these results demonstrate that EGFR overexpression confers additional survival signals in the breast cancer cells that suppress the apoptotic-inducing effect caused by loss of FAK function.

**Inhibition of FAK and EGFR in BT474-EGFR cells downregulates both ERK1/2 and TNFR family dependent AKT survival pathways.**

Since EGFR appeared to function as a survival signal to protect breast cancer cells from apoptosis induced by FAK downregulation, we investigated the downstream biochemical pathways for EGFR and FAK, beginning with the MAP kinase pathways. After 24 hours of infection with Ad-FAK-CD, the BT474-EGFR cells demonstrated upregulation of phosphorylated ERK1/2 that could be inhibited by the EGFR kinase inhibitor AG1478 (Fig. 5). This high level of ERK1/2 phosphorylation was not seen the BT474-EGFR cells treated with control Ad-LacZ adenovirus or in the pcDNA3 vector control cells treated with Ad-FAK-CD, showing that Ad-FAK-CD initiated an EGFR-mediated stress response and survival signaling that includes ERK1/2 phosphorylation.

We also examined the AKT (Protein kinase B) pathway (47), because FAK has been shown to act upstream of this serine/threonine kinase, which has important survival signal
functions in tumor cells (48). AKT is constitutively expressed and serine-473-phosphorylated in BT474-EGFR, as well as control BT474-pcDNA3 cells treated with control LacZ adenovirus (Fig. 5). BT-474-pcDNA3 cells treated with Ad-FAK-CD down-regulated AKT, as total and serine-473-phosphorylated AKT were not present in these cells (Fig. 5, AKT panels, lanes 3). In contrast to the BT474-pcDNA3 control cells, BT474-EGFR cells (independent of EGF ligand) expressed AKT and serine-473-phosphorylated the active form of AKT in response to downregulation of FAK (Fig. 5). However, when the EGFR kinase activity was inhibited by AG1478 in the BT474-EGFR cells, there were undetectable levels of AKT protein by Western blot and AKT was completely dephosphorylated (Fig. 5). Based on these data, EGFR not only signals through ERK1/2, but also has an effect on the ability of AKT to resist downregulation in response to FAK inhibition.

To further assess the relationship of EGFR overexpression and FAK downregulation to the ERK1/2 and AKT pathways in the BT474 cells, we inhibited TNF receptor (TNFR) family signaling in the BT474-EGFR and pcDNA3 cells, based on our recent data that the TNFR family regulates FAK-CD induced apoptosis (2). In order to analyze if these receptors are important in reduction of AKT protein levels in apoptotic BT474-pcDNA3 cells infected with Ad-FAK-CD, we blocked death receptor pathways by co-infection of cells with adenoviral dominant-negative FADD, [(Fas-associated death domain protein, ΔFADD, lacking amino acids 1-79 of the death effector domain (49)]. Under these conditions, where the death complex was inhibited, AKT protein levels were not reduced and AKT was serine-473 phosphorylated in BT474-pcDNA3 and BT474-EGFR cells (Fig. 5, two right lanes). The results demonstrate that AKT downregulation (reduction of protein level) is mediated through a TNFR family pathway in BT474-pcDNA3 cells infected with Ad-FAK-CD. In
contrast, dominant negative FADD had no effect on ERK1/2 phosphorylation in the cells, indicating the independence of the ERK1/2 survival pathway from the TNF receptor family pathways (Fig. 5). These results show that TNF receptor family signaling, which is involved in FAK-CD induced apoptosis, is also important in downregulation/cleavage of AKT.

Taken together, these biochemical results parallel the cell biological results above, whereby inhibition of both FAK and EGFR caused both the highest level of apoptosis as well as inhibition of both the MAP kinase (ERK1/2) and TNF receptor family dependent AKT signaling pathways.

**EGFR overexpression protects p125\textsuperscript{FAK}, caspase 3 and caspase 8 from complete degradation in response to FAK inhibition in BT474 cells reversed by AG1478.**

To further examine the effects of EGFR on resistance to apoptosis, we tested whether overexpression of EGFR would protect the endogenous p125\textsuperscript{FAK} in the breast cancer cells from degradation in response to Ad-FAK-CD. Previous work from our group has shown that p125\textsuperscript{FAK} was degraded 24 hours after Ad-FAK-CD infection in parental BT474 cells, and this effect was mediated through caspases 8 and 3 (2). Similarly, in these studies, the p125\textsuperscript{FAK} protein was degraded in the control BT474-pcDNA3 cells upon infection with Ad-FAK-CD (Fig. 5). However, in BT474-EGFR cells, EGFR overexpression protected p125\textsuperscript{FAK} from complete degradation by Ad-FAK-CD, as shown by the 125 kDa FAK band that was present in BT474-EGFR cells but not in the BT474-pcDNA3 cells (Fig. 5, lanes 3-5). Downregulation of both FAK and EGFR did lead to complete degradation of p125\textsuperscript{FAK} in the BT474-EGFR cells, whereby probing the Western blots with an antibody to the N-terminus
of FAK detected only 85-90 kDa degradation products (Fig. 5, lanes 6 and 7). Co-infection of BT474 cells with Ad-FAK-CD and Ad-ΔFADD, blocked cleavage of FAK (Fig. 5).

In BT474-pcDNA3 cells, treatment with Ad-FAK-CD induced caspase-3 activation and PARP (caspase-3 substrate) cleavage (Fig. 6A). Pretreatment of these cells with caspase-3 family inhibitor, Ac-DEVD-CHO peptide, prior to infection with Ad-FAK-CD blocked activation of caspase-3 and cleavage of PARP (Fig. 6A) and increased the level of total AKT and FAK (Fig. 6A), indicating that reduction of AKT and FAK protein levels is the result of FAK and AKT cleavage by a caspase 3 pathway in the BT474 cells.

In contrast, overexpression of EGFR protected caspases 3 and 8 from degradation caused by FAK down-regulation. In the BT474-EGFR cells treated with Ad-FAK-CD, there was significant protection of caspase 3 from cleavage, with incomplete PARP cleavage (Fig. 6B, lanes 4 and 5). However, when EGFR was inhibited under these conditions, the cleavage of caspases 3 and PARP were restored to equivalent levels as the BT474-pcDNA3 cells (Fig. 6B, lanes 3, 6, and 7). The upstream caspase 8 showed a similar effect, whereby the BT474-EGFR cells did not activate caspase 8 in response to FAK-CD, but in combination with EGFR inhibition, cleaved the inactive proform and activated the enzyme (Fig. 6C). Co-infection of BT474-pcDNA3 cells with Ad-FAK-CD and Ad-ΔFADD, protected caspase-8, caspase-3 and PARP from cleavage (Fig. 6).

**Dual inhibition of FAK and EGFR in the BT-20 breast cancer cell line, endogenously overexpressing EGFR, enhances apoptosis, downregulating AKT and ERK1/2 survival pathways.**

In a final series of experiments, we tested whether endogenous EGFR in a breast cancer cell line would have similar survival signal effects as our model system of EGFR
overexpression in the BT474-EGFR cells. We used the BT20 cell line that has been shown to express high levels of endogenous EGFR (44).

Similar to the BT474-EGFR cells, inhibition of FAK by Ad-FAK-CD in the BT 20 breast cancer cells induced loss of adhesion, although this effect was seen at later time points, 46-71 hours after adenovirus infection. BT 20 cells treated with Ad-FAK-CD started to detach at 46 hours, and greater than 60% of cells treated with FAK-CD minus or plus EGF or cells treated with FAK-CD plus AG1478 (± EGF) detached by 71 hours (Fig. 7A).

However, inhibition of FAK and EGFR enhanced the levels of apoptosis in these cells (Figure 7B) in a similar fashion as seen in the BT474-EGFR cells (Fig. 4B). At 46 hours after infection, the levels of apoptosis were slightly increased when cells were treated with FAK-CD and AG1478, compared to cells that had been treated with FAK-CD (7% versus 1.6%). However, at 71 hours, this effect was more apparent, where the apoptotic rate with FAK-CD alone was 10%, but addition of the EGFR kinase inhibitor increased the rate to 33% and 43% (- EGF and +EGF respectively) (Fig. 7B).

Next, we directly compared the biochemical effects of FAK and EGFR inhibition between the BT474-EGFR cells and the BT-20 cells. As shown by control Ad-LacZ infection, the levels of endogenous EGFR and p125FAK expression, as well as the levels of AKT phosphorylation were higher in the BT20 cells (Fig.8A, lanes 6) than in BT474-pcDNA3 cells (lane 1) or the BT474-EGFR cells (lane 2). After 24 hours of infection with Ad-FAK-CD, the BT-20 cells totally protected FAK from degradation detected in BT474-pcDNA3 cells (Fig.8A). The ERK1/2 survival pathway was activated in BT20 cells, as in BT474-EGFR cells, as ERK1/2 was highly phosphorylated in BT20 cells infected with Ad-FAK-CD and dephosphorylated upon treatment with AG1478. However, AKT remained
highly active, serine-473 phosphorylated in BT20 cells after 24 hours of FAK-CD infection, as BT20 cell have high levels of AKT (Fig. 8A). At 72 hours after FAK-CD expression and in the presence of AG1478, BT20 cells detached and had down-regulated both ERK1/2 and AKT phosphorylation, and also downregulated total p125FAK, caspase-3, and AKT proteins (Fig. 8B).

Next, we tested whether the degradation of FAK and AKT in the BT20 cells was caspase 3-dependent, similar to the BT-474 cells. To downregulate FAK and AKT more efficiently in BT20 cells, we used the same dose of Ad-FAK-CD per cell but concentrated it 3.5 times in the culture medium. Under these conditions, FAK and AKT were completely downregulated at 55 hours (Fig. 8C). However, pretreatment of cells with caspase-3 subfamily inhibitor (DEVD-CHO) blocked degradation of FAK and AKT (Fig. 8C), demonstrating that FAK and AKT downregulation in AG1478 and Ad-FAK-CD-treated BT20 cells was caspase-3 dependent.

These results mechanistically support increased levels of apoptosis seen with Hoechst staining in the detached cells, treated with Ad-FAK-CD and AG1478 and show that dual inhibition of EGFR and FAK increased apoptosis in BT20 cell line, inhibiting the same survival signaling pathways as BT474-EGFR cells.

**DISCUSSION.**

These studies demonstrate the cooperativity of both FAK and EGFR signals in suppressing apoptosis in breast cancer cells. While there appears to be a physical association of these tyrosine kinases, their individual survival signals appear also in part, to be in parallel. Thus, inhibition of both FAK and EGFR signaling pathways led to significantly
higher levels of apoptosis than inhibition of either one alone. These results were supported from a biochemical level, whereby the EGFR-overexpressing cells had increased levels of both ERK1/2 and AKT phosphorylation and did not demonstrate complete p125$^{FAK}$, caspase-3, or caspase-8 degradation until both FAK and EGFR signaling had been interrupted. Thus, this study is the first to show the cooperative effect of FAK and EGFR inhibitors in induction of apoptosis in human breast cancer cells. We propose a model of survival signaling in breast cancer cells whereby FAK and EGFR overexpression can promote survival signals via an AKT-dependent mechanism as well as via an ERK1/2 pathway (Figure 9). Dual inhibition of FAK and EGFR led to apoptosis via death receptor mediated signaling.

Individually, both FAK and EGFR have been shown to be overexpressed in human breast cancer specimens (15,17,18). However, the relationship between these kinases and the subsequent cellular effects in breast cancer remain unclear. It has been shown that FAK and EGFR can associate when co-expressed in FAK-/- fibroblasts in the presence of EGF, suggesting that FAK can mediate a linkage between growth factor receptors and integrins (27). Similarly, the association of FAK and EGFR has been shown in A431 epidermoid cancer cells (26) and A549 adenocarcinoma cells (22), both of which express extraordinarily high levels of EGFR (50,51). However, these studies differed in whether FAK and EGFR constitutively associate (26), or whether this association requires EGF ligand (22). Our results support a constitutive association between FAK and EGFR in BT474 breast cancer cells stably overexpressing EGFR, but it is unclear what effect this association has on downstream signaling pathways.

The studies of FAK and EGFR in cancer cells have largely focused on their effects on tumor cell motility. Inhibition of FAK in A549 cells was shown to inhibit EGF stimulated
motility, providing further evidence that FAK can integrate motility signals from EGF to EGFR (22). Other motility studies suggest that FAK is dephosphorylated in response to EGF, promoting tumor invasion and motility (26). In studies of human glioblastoma cells, inhibition of FAK function by exogenously expressing the focal adhesion targeting domain also diminished EGFR-directed cell motility (19). We have examined motility of the breast cancer cells in our system, and found that downregulation of FAK rapidly diminished both random and EGF-directed cell motility (data not shown), supporting the hypothesis that FAK is involved in EGF-directed motility pathways. Thus, the model proposed by Hauck et al. (22), is consistent with our findings in breast cancer cells. In their model, interactions between FAK and EGFR and FAK and integrins, concomitant with Src family activity via FAK phosphorylation at Tyr-397, activate downstream pathway that promote motility.

However, it appears that the effects of FAK overexpression in breast cancer cells are not simply limited to motility and invasion, but can cooperate with EGFR signaling to suppress apoptosis and enhance survival of breast cancer cells. Studies of FAK in primary breast cancer specimens have shown that upregulation of FAK expression is an early event in tumorigenesis, occurring in ductal carcinoma \textit{in situ}, before the tumor has developed the capacity for invasion and metastasis (18). These observations support the hypothesis that FAK functions to promote survival during tumor cell proliferation before invasion and migration have occurred. Furthermore, other studies in breast cancer cell lines suggest that FAK has two separate functions in human tumor cells: one promoting adhesive interactions between tumor and matrix, and the other providing survival signals to resist apoptosis (2). Our results in this study also support this hypothesis, whereby downregulation of FAK function had effects on apoptosis in EGFR-overexpressing breast cancer cells.
In these studies, the biochemical mechanisms of apoptotic resistance appeared to involve both the ERK and AKT pathways. Overexpression of EGFR was associated with a robust phosphorylation of ERK1/2 in the BT474-EGFR cells, and this appeared to augment the resistance to apoptosis induced by FAK downregulation. This is consistent with other studies implicating the ERK pathways in apoptotic resistance, including TNF-α induced apoptosis in fibrosarcoma cells (52), and stress-induced apoptosis in A431 cells (53). In the latter study, Src-dependent phosphorylation of EGFR led to ERK activation and the induction of survival signals in response to UV irradiation (53). Other investigators have implicated EGFR activation in keratinocyte survival by sustained MEK/MAPK signaling activation (54). Intriguingly, EGFR has been shown to transmit a survival signal to MAPK, even in the absence of EGFR kinase activity (55), implicating other kinases such as the Src family in this pathway. Nonetheless, the persistent phosphorylation of ERK1/2 in our studies also was associated with the inability of FAK inhibition to cause downregulation and dephosphorylation of AKT in EGFR overexpressing cell lines. This suggested that EGFR also was having a survival signal function through this serine/threonine kinase. Furthermore, this effect appeared to be dependent on EGFR kinase activity, as AG1478 abrogated the protection of AKT. In fact, several studies have shown that EGFR can signal directly to AKT via PI-3 kinase (42,56-58). In a model of oxidative stress-induced apoptosis, H₂O₂ activated AKT through an EGFR/PI-3-K-dependent pathway (42). Similarly, activation of EGFR signaling in T47D breast cancer cells and HEK293 cells protected these cells lines from Fas-induced apoptosis via an AKT-dependent mechanism (56). These observations have recently been extended to TRAIL-induced apoptosis, whereby signaling from EGFR to AKT protected HEK293 and MDA MB 231 cells from apoptosis by inhibiting mitochondrial
cytochrome c release (57). In our system, the apoptosis induced by FAK downregulation appeared to function through similar receptor-mediated apoptotic pathways, consistent with our previous studies (2). In addition, inhibition of the death complex with dominant negative FADD inhibited degradation of AKT, independent of ERK activation, suggesting that the effects of FAK inhibition in these cells were mediated through a TNF receptor family mediated, AKT-dependent pathway (Figure 9). Furthermore, TNF receptor family FADD-dependent AKT cleavage was recently reported in MDCK epithelial cells (59). Ad-FAK-CD may induce death receptor by activating death domain containing proteins such as Fas, TNFR, or DR/Trail proteins or by affecting adapter proteins such as FADD, TRADD or RIP. These events appeared to activate the downstream caspase-8 and caspase-3 cascade with cleavage of important survival proteins as AKT and FAK (Fig. 9). In EGFR and FAK-positive cells, binding of EGFR to FAK may partially block FAK-CD-induced apoptosis while EGFR itself can signal directly to AKT and ERK1/2. Furthermore, EGFR survival signaling was kinase-dependent, as inhibition of EGFR with AG1478 induced Ad-FAK-CD apoptosis (Figure 9). In addition, the magnitude of these effects was cell type specific, as breast cancer cells such as BT20 that express high levels of FAK, EGFR, and AKT were more resistant to inhibition of these pathways than BT474 cells that expressed lower levels of these survival proteins.

Our model of survival signaling in the BT474-EGFR and BT-20 breast cancer cells is that there are multiple cross-talking signaling pathways via ERK and AKT that augment the resistance of tumor cells to the apoptotic-promoting effects of tumor dissemination. The persistent signaling to AKT and MAP kinase pathways, contributes to the resistance to apoptosis in these cells that overexpress EGFR. From these studies we conclude that FAK
and EGFR cooperatively suppress apoptosis in breast cancer cells, suggesting that targeting both signaling pathways will have an enhanced apoptotic effect in breast cancers that overexpress these kinases.

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    274, 17612-17618

    Cancer Res 62, 488-496


    Biol Chem 276, 34702-34707
FIGURE LEGENDS.

Figure 1. BT474-EGFR cells: EGF-dependent signaling and its inhibition by AG1478.

(A) Western blotting was performed with monoclonal anti-EGFR antibody in the vector control BT474-pcDNA3 cells and the BT474-EGFR cells. Equal protein loading was analyzed by Western blotting with anti-β-actin antibody.

(B) EGFR increases phosphorylation after EGF stimulation and is inhibited in the presence of AG1478. Immunoprecipitation (IP) was done with monoclonal anti-EGFR antibody, followed by the Western blotting (WB) with anti-phosphotyrosine RC20 antibody. Cells were treated with EGF (10ng/ml) for 10 min after overnight serum starvation. AG1478 (EGFR kinase inhibitor) was used at 5µM for 15 min before EGF stimulation.

(C) Total phosphorylation of proteins. BT474 cells were serum starved overnight and induced by EGF (10ng/ml) for 10 min without or with AG1478 (5µM) pretreatment for 15 min. The human EGFR-overexpressing epidermoid carcinoma cell line, A431, was used as a positive control for induction of EGFR phosphorylation. Western blotting was performed on total cell lysates with anti-phosphotyrosine antibody, RC20. Western with anti-β-actin on BT-474 lysates was performed to confirm equal protein loading.

(D) ERK1/2 activation in response to EGF treatment, reversed by AG1478.

ERK1/2 activation was analyzed with phospho-specific ERK1/2 antibody after EGF stimulation for 10 min and with AG1478 pretreatment after overnight serum-free medium starvation. Total ERK1/2 level was determined with anti-ERK1/2 polyclonal antibody. P-ERK1/2 shows phosphorylated active ERK1/2.
Figure 2. FAK constitutively associates with EGFR in BT74-EGFR cells.

A. BT474 cells were stimulated with EGFR as described in Figure 1. Left panel: IP: FAK: Immunoprecipitation (IP) was performed with anti-FAK monoclonal antibody 4.47, and Western blotting was done with anti-phosphotyrosine antibody. The membrane was stripped and Western blotting was performed with anti-FAK antibody, and then stripped again and reprobed with anti-EGFR antibody to show that FAK co-precipitated with EGFR. The same experiment as above was performed with immunoprecipitation of EGFR that detected FAK and EGFR association (Right panel). The experiment was done for three times with the same results. The image is a composite from the same film/gel.

B. FAK and EGFR association and co-localization at focal adhesions is detected by co-immunostaining. Immunostaining was performed with primary mouse monoclonal anti-FAK (4.47) antibody and probed with Rhodamine-conjugated anti-mouse secondary antibody. After washing, dual immunostaining was performed with anti-EGFR monoclonal antibody and probed with FITC-conjugated secondary anti-mouse antibody. Merged image was obtained with Adobe Photoshop 6.0 program.

Figure 3. Adenoviral-mediated expression of FAK-CD and LacZ in BT-474 cells.

BT474 cells were infected with Ad-LacZ and Ad-FAK-CD, as described in Materials and Methods. In titration experiments, the viral titer was detected with maximal cell infectivity. At optimal viral concentration (500 ffu/cell) approximately 100% of cells were blue-X-gal positive determined by X-gal staining for Ad-LacZ infection (A) and HA-positive cells were determined by immunostaining with anti-HA antibody for Ad-HA-tagged FAK-CD infection after 24 hours of infection (B). Right upper panel: Detached cells were stained with Bodipy-
phallacidin-FITC that stains actin inside cells for better cell visualization of cells under fluorescent microscope. Right lower panel: HA-Rhodamine immunostaining shows that 100% of cells expressed HA-FAK-CD. LacZ positive cells were captured under 40x magnification and HA-FAK-CD expressing cells that have round morphology after 24 hours were captured under 100x magnification on fluorescent microscope.

**Figure 4.** BT474 cells overexpressing EGFR suppressed apoptosis but not cell detachment induced by FAK downregulation.

**(A) Ad-FAK-CD infection stimulated cell detachment in BT474 cells.** BT474-pcDNA3 and BT474-EGFR cells were infected with Ad-FAK-CD or Ad-LacZ (Materials and Methods) at 500 ffu/cell, that resulted in 100% cell infectivity. After 24 hours, Ad-FAK-CD-infected detached cells were counted on a hemocytometer. Ad-LacZ infected cells were resistant to detachment and were not apoptotic (not shown). Four independent experiments were done with the same result, and a representative experiment is shown. The mean percent of detached cells is shown ± standard deviations from three independent cell counts on hemocytometer.

**(B) Apoptosis induced by Ad-FAK-CD is inhibited by EGFR overexpression and reversed by EGFR-kinase inhibitor AG1478.** Detached BT474 cells that were infected with Ad-FAK-CD were fixed and analyzed for apoptosis. Apoptosis was determined by Hoechst staining. Ad-LacZ infected cells were not apoptotic (not shown). Bars represent mean values ± standard deviations. More than 100 cells were counted from three independent fields for each experimental treatment in three independent experiments.
(C). Hoechst staining of BT474 cells treated with Ad-FAK-CD. Apoptotic Ad-FAK-CD infected cells with fragmented nuclei have bright Hoechst staining of condensed nuclear chromatin. Ad-LacZ infected cells did not undergo apoptosis and had unfragmented nuclei, as control BT474 cells (not shown). Left panel: Cells viewed for phase contrast. Right panel: Identical cells viewed for Hoechst stained nuclei. Normal cells had faintly stained nuclei while apoptotic condensed nuclei stained brightly.

(D) ERK1/2 inhibitor, PD98059 increases Ad-FAK-CD induced apoptosis in BT474-EGFR cells.

BT474-EGFR cells were pretreated with 10 µM of PD98059 for 15 min before Ad-FAK-CD treatment. PD98059 was present during adenoviral incubation for 24 hours. After 24 hours of adenoviral infection, apoptosis was measured as in Fig. 4B. Upper panel: PD98059-increased apoptosis in BT474-EGFR cells. Bars represent mean values ± standard deviations. More than 100 cells were counted from three independent fields for each experimental treatment in three independent experiments. Statistical significance was determined using Student’s t test. *, a significant difference from pcDNA3 control, P< 0.02, **, a significant difference from EGFR sample, P< 0.04. Lower panel: Hoechst stained nuclei are shown.

Figure 5. EGFR-overexpressing BT474 cells infected with Ad-FAK-CD activate ERK1/2 and AKT signaling pathways and partially protect FAK from degradation. Ad-ΔFADD blocks FAK degradation and AKT downregulation caused by Ad-FAK-CD. BT474-pcDNA3 cells and EGFR-overexpressing BT474-EGFR cells were treated with either control Ad-LacZ or Ad-FAK-CD, with or without EGFR kinase inhibition by AG1478 and analyzed for ERK1/2 and AKT activation and FAK degradation. ERK1/2 activation was
analyzed by Western blotting with anti-ERK1/2 phospho-specific antibody and with anti-total ERK1/2 antibody. ERK1/2 was activated in BT474-EGFR cells infected with Ad-FAK-CD (+/- EGF) and inhibited by AG1478 treatment. AKT status was analyzed with phospho-serine-473-specific anti-AKT antibody and with anti-total AKT antibody. AKT is not detected in BT474-pcDNA3 cells infected with Ad-FAK-CD, but detected in control cells infected with Ad-LacZ. BT474-EGFR cells expressed active serine-phosphorylated and total AKT after Ad-FAK-CD infection. Treatment with AG1478 (±EGF) caused downregulation of AKT. Ad-ΔFADD protected AKT from downregulation and FAK from complete degradation. Western with anti-HA antibody controlled for HA-FAK-CD protein level in the samples. Equal protein loading was controlled with anti-α-tubulin antibody. Each experiment was performed five times with different adenoviral preparations with the same results and a representative experiment is shown.

Figure 6. Caspase 3-dependent degradation of FAK and AKT in BT-474 cells.
A. Caspase-3 inhibitor, Ac-DEVD-CHO blocks Ad-FAK-CD-induced activation of caspase-3 and PARP cleavage and degradation of FAK and AKT in BT-474-pcDNA3 cells. BT474-pcDNA3 cells were pretreated with caspase-3 family inhibitor (Ac-DEVD-CHO, at concentration 50 µM (2) for 1 hour before adenoviral infection. After Ad-FAK-CD infection for 24 hours, cells were collected and analyzed for FAK and AKT protein levels, as in Fig. 5. Caspase-3 activation was determined with anti-caspase-3 antibody, specific to inactive, 32 kDa pro-caspase-3 protein. Caspase-3 substrate, PARP, cleavage analysis was performed by Western blotting with anti-PARP antibody, specific to uncleaved 116 kDa PARP. The image is a composite from the same film, lanes 1 and 2.
Figure 6 B, C. Activation of caspases 3 and 8 and PARP in response to Ad-FAK-CD is blocked in BT-474-EGFR cells and reversed by inhibition of EGFR.

(B) Ad-FAK-CD induced activation of caspase-3 cleavage and PARP cleavage in BT474 is blocked in BT474-EGFR cells and in Ad-ΔFADD-BT474 cells. Caspase-3 activation was determined with anti-caspase-3 antibody. BT474-EGFR cells and Ad-ΔFADD cells suppressed caspase-3 activation. PARP cleavage analysis was performed by Western blotting with anti-PARP antibody, specific to uncleaved 116 kDa PARP on the same lysates as in upper panel. Loading was controlled with anti-α-tubulin antibody. Each experiment was repeated three times with two independent Ad-FAK-CD adenoviruses with the same results and a representative experiment is shown.

(C) Activation of caspase-8 cleavage in BT474 cells infected with Ad-FAK-CD is blocked by EGFR overexpression and by Ad-ΔFADD expression. Caspase-8 activation was determined with anti-caspase-8 antibody, specific to uncleaved inactive 55 kDa caspase-8. BT474-EGFR cells and Ad-ΔFADD cells suppressed caspase-8 activation. Beta-actin protein was used for normalization of protein level.

Figure 7. BT-20 breast cancer cells, endogenously overexpressing EGFR, show detachment and increased apoptosis with FAK and EGFR inhibition.

(A) Detachment assay was done as in BT474 cells (Fig. 4A). BT20 cells started to detach at 46 hours after Ad-FAK-CD infection with >60% cells detached at 71 hours. At 71 hours cells were equally detached in all treated samples. Cells did not detach after Ad-LacZ infection (not shown). (B) At 71 hours apoptosis was increased in BT20 cells by FAK and EGFR
inhibition, with and without EGF. Apoptosis was determined as in Fig. 4B. Two independent experiments were done with the same result, and a representative experiment is shown.

Figure 8. Endogenous overexpression of EGFR protects FAK from degradation and activates AKT and ERK1/2 survival pathways after FAK-CD expression reversed by AG1478 inhibitor.

A. FAK and p-Ser473AKT levels 24 hours post Ad-FAK-CD infection and AG1478 treatment. Western blotting with anti-EGFR, AKT, and FAK antibodies were performed as described in Fig. 5. The BT20 cell line did not degrade FAK at 24 hours after Ad-FAK-CD infection. BT20 cells have highly active AKT and ERK1/2 after FAK-CD infection. HA-tagged FAK-CD expression level was analyzed with anti-HA-antibody.

B. FAK, p-Ser-AKT, p-ERK1/2 and caspase-3 levels 72 hours post Ad-FAK-CD infection and AG1478 treatment. BT20 cells had less FAK, AKT (total and p-Ser-473) and p-ERK1/2 protein levels at 72 hours of Ad-FAK-CD infection in AG1478 treated samples. p-ERK1/2 (Phosphorylated ERK1/2) and ERK1/2 samples were run on a parallel gel with FAK and AKT samples and a composite image from the same film is shown for ERK samples. Protein levels were analyzed as in (A) with the same antibodies.

C. Downregulation of FAK and AKT in Ad-FAK-CD and AG1478-treated BT20 cells are caspase-3 family dependent. BT20 cells were pretreated first with cell-permeable caspase-3 family inhibitor (DEVD-CHO) at concentration 5 µM for 1 hour and then with AG1478 at concentration 5 µM and infected with Ad-FAK-CD. Ad-FAK-CD was added at the same viral dose as in Fig. 8 A, B, but was 3.5 times more concentrated (as added in a 3.5 times less volume of medium for better infectivity and faster apoptotic response).
Caspase-3 and AG1478 inhibitors were present during adenoviral incubation, added fresh at the same doses every 24 hours. Cells were collected at 55 hours after adenoviral infection. Western blotting was performed with caspase-3, PARP, FAK and AKT antibodies as described in Fig. 6A. Equivalent amounts of total protein from cell lysates were loaded on the gel.

**Figure 9. Model of FAK/EGFR-dependent apoptosis in breast cancer cells with high levels of FAK and EGFR expression.**
Figure 2 B

BT474-EGFR cells

FAK-Rhodamine

EGFR-FITC

Merged (FAK-Rhodamine + EGFR-FITC)

BT 20 cells

FAK-Rhodamine

EGFR-FITC

Merged (FAK-Rhodamine + EGFR-FITC)
Figure 3

A

X-gal staining

BT474 cells - No Adeno-LacZ

BT474 cells + Adeno-LacZ

B

HA-staining

BT474-pcDNA3 + Adeno-HA-FAK-CD

Actin-FITC

HA-FAK-CD-Rhodamine
Figure 4 A, B

A

![Graph A]

Detachment (%)

**BT-474 cells + Adeno-FAK-CD**

B

![Graph B]

Apoptosis (%)

**BT474 cells + Adeno-FAK-CD**
Figure 4D

![Graph showing apoptosis (%) with different treatments: pDNA3, EGFR, EGFR+EGF, EGFR+AG1478, EGFR+PD98059.](graph)

**BT-474 cells + Ad-FAK-CD**

**BT474-EGFR + Ad-FAK-CD**

***Phase***

- PD98059

***Hoechst***

- PD98059

Downloaded from http://www.jbc.org by guest on September 1, 2017
Figure 6 A

BT474-pcDNA3 cells

Ad-FAK-CD

Ad-Lac Z  Caspase-3 Inh.  Ac-DEVD-CHO

Total FAK  WB: FAK

Total AKT  WB: AKT

Inactive caspase-3  WB: Caspase-3

Uncleaved PARP  WB: PARP

α–Tubulin  WB: α–Tubulin
**Figure 6 B, C**

**B**

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**Inactive Caspase-3 (32 kDa)**

**Uncleaved PARP (116 kDa)**

**C**

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**Inactive caspase-8 (55 kDa)**
Figure 7 A, B

A

![Graph showing Detachment (%) for different treatments over time (46 h and 71 h). The treatments include FAK-CD, FAK-CD+EGF, FAK-CD+AG1478, and FAK-CD+EGF+AG1478. BT20 cell line + Adeno-FAK-CD.]

B

![Graph showing Apoptosis (%) for different treatments over time (46 h and 71 h). The treatments include FAK-CD, FAK-CD+EGF, FAK-CD+AG1478, and FAK-CD+EGF+AG1478. BT20 cell line + Adeno-FAK-CD.]
FAK AND EGFR POSITIVE BREAST CANCER CELLS

FAK-CD + AG1478

DEATH RECEPTORS

CASPASE-8

CASPASE-3

FAK-CD + PD98059

AKT

DOWNREGULATION

P-Ser-473 DEPHOSPHORYLATION
CLEAVAGE

CASPASE-3 INHIBITOR
(Ac-DEVD-CHO)

DEATH RECEPTOR INHIBITOR
(ΔFADD)

FAK

EGFR

ERK1/2

APOPTOSIS
Dual inhibition of focal adhesion kinase (FAK) and epidermal growth factor receptor (EGFR) pathways cooperatively induces death receptor-mediated apoptosis in human breast cancer cells

Vita Golubovskaya, Lucia Beviglia, Li-Hui Xu, H. Shelton Earp III, Rolf Craven and William Cance

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