Dual Inhibition of Focal Adhesion Kinase and Epidermal Growth Factor Receptor Pathways Cooperatively Induces Death Receptor-mediated Apoptosis in Human Breast Cancer Cells*

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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 EGFR-overexpressing BT474 cells. In low EGFR-expressing BT474-pcDNA3 vector control cells, inhibition of FAK by the FAK C-terminal domain caused detachment and apoptosis via pathways involving activation of caspase-3 and -8, cleavage of poly(ADP-ribose) polymerase, and caspase-3-dependent degradation of AKT. This apoptosis could be rescued by the dominant-negative Fas-associated death domain, indicating involvement of the death receptor pathway. EGFR overexpression did not inhibit detachment induced by the FAK C-terminal domain, but did suppress apoptosis, activating AKT and ERK1/2 survival pathways and inhibiting cleavage of FAK, caspase-3 and -8, and poly(ADP-ribose) polymerase. 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 (BT20) endogenously overexpressing these kinases also induced apoptosis via the same mechanism as in the EGFR-overexpressing BT474 cells. The results of this study indicate that dual inhibition of FAK and EGFR signaling pathways can cooperatively enhance apoptosis in breast cancers.

The invasion and metastasis of cancer require a controlled process of basement membrane degradation, cell motility, and anchorage-independent cell 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 has recently been shown to modulate apoptotic signaling is the focal adhesion kinase (FAK)1 (1–3). This non-receptor protein-tyrosine kinase localizes to points of cell contact with the extracellular matrix, the focal adhesions (4, 5).

FAK was originally isolated as a tyrosine-phosphorylated 125-kDa protein in v-Src-transformed chicken embryonic fibroblasts (6, 7). FAK includes an N-terminal domain with a primary autophosphorylation site that directs interaction with the Src homology-2 domain (8); a central catalytic domain with two major sites of phosphorylation (Tyr576/Tyr577); and a C-terminal domain with two proline-rich segments and a focal adhesion targeting subdomain, which binds paxillin, talin, and other proteins (4, 9). FAK activity is regulated by extracellular matrix receptors and integrins and involved in cellular processes such as spreading, motility, proliferation, and survival (4, 10). A non-catalytic domain of FAK, FAK-related nonkinase (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 causes dephosphorylation of FAK at Tyr297 (13) and blocks FAK-mediated fibroblast migration (14).

FAK was shown to be overexpressed compared with 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 focal adhesion targeting subdomain leads to cell rounding, detachment, reduction of invasion, and apoptosis (1, 19–22). 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 prevent anoikis and stimulate transformation of epithelial cells, resulting in anchorage-independent growth and tumor formation in nude mice (24). Further evidence for the anti-apoptotic role of FAK was shown in the leukemic cell line HL-60, where FAK is associated with activation of NF-κB and inhibition of caspase-3 (24). Conversely, caspase-3 and -6 may promote apoptosis in

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§§The abbreviations used are: FAK, focal adhesion kinase; FRNK, FAK-related nonkinase; EGFR, epidermal growth factor; EGFR, epidermal growth factor receptor; FAK-CD, FAK C-terminal domain; ERK, extracellular signal-regulated kinase; TNFR, tumor necrosis factor receptor; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; Ad, adenovirus; FADD, Fas-associated death domain; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; TUNEL, terminal deoxynucleotidyltransferase-mediated biotinylated UTP nick end labeling; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase.
part by cleaving FAK and generating a C-terminal FRNK-like peptide (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 170-kDa EGFR, receptor homo- and heterodimerization is promoted, activating receptor tyrosine kinase activity (28) and downstream signaling (reviewed in Refs. 29–32). 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 (32, 40) and may promote proliferation, migration, invasion, and cell survival as well as inhibition of apoptosis (41–43). Recent reports have suggested that FAK serves to integrate EGFR signals upon EGF induction, promoting tumor cell motility and invasion (22, 27). 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 a noninvasive to an invasive and metastatic tumor phenotype is unknown, and their cooperation in preventing apoptosis has not been mechanistically examined.

In this 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 the FAK C-terminal domain (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 an EGFR kinase inhibitor. Dual inhibition of FAK by FAK-CD and of EGFR by AG1478 cooperatively enhanced apoptosis in human breast cancer cell lines via inhibition of signaling that involved both tumor necrosis factor receptor (TNFR) family-dependent AKT and ERK1/2 pathways. This is the first report of the role of FAK and EGFR in apoptosis showing 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 the BT474 cell line used in this study had low expression of EGR (Her-1) and Her-2. BT20 breast carcinoma cells, which overexpress EGFR (44), were maintained in Eagle’s minimal essential medium containing 10% fetal bovine serum. Cell lines were incubated at 37°C in a 5% CO2 humidified incubator. For EGF stimulation (Western blotting and immunoprecipitation), cells were serum-starved overnight in serum-free medium. In FAK-CD-induced apoptosis experiments, EGFR was added three times at 0, 6, and 20 or 22 h of incubation (Western blotting and immunoprecipitation), cells were serum-starved in 0.1% serum. The clone of the BT474 cell line used in this study had low FAK expression and activity during progression from a noninvasive to an invasive and metastatic tumor phenotype is unknown, and their cooperation in preventing apoptosis has not been mechanistically examined.

**Staining with X-Gal**—Cells were infected with Ad-lacZ as described above. Briefly, 24 h after infection, cells were fixed for 10 min on ice with 2% formaldehyde and 0.2% glutaraldehyde in 1× phosphate-buffered saline (PBS). After washing twice with 1× PBS, cells were stained for 1–3 h with 1 μg/ml X-gal in X-gal buffer (5 mM K4Fe(CN)6, 5 mM K3Fe(CN)6, and 2 mM MgCl2 in 1× PBS). X-gal-positive (blue) cells were counted for determining of infection efficiency.

**Stable BT474-EGFR and BT474-pcDNA3 clones were obtained using RPMI 1640 medium with the selective antibiotic Geneticin (500 μg/ml; G418, Invitrogen). 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 1× PBS and lysed on ice for 30 min in buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 50 mM NaF, 1 mM NaVO3, 10% glycerol, and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, and 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 a Bio-Rad kit. The cleared lysates with equivalent amounts of protein were incubated with 5 μl of primary antibody for 1 h at 4°C and with 25 μl of protein A/G-agarose beads (Oncogene Research Products Inc.). The precipitates were washed three times with the lysis buffer and resuspended in 30 μl of 2× Laemmli sample buffer. Lysates were boiled briefly and then subjected to SDS-10% polyacrylamide gels (Bio-Rad). The phosphorylation status of the proteins examined was detected with horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20, and anti-caspase-3 and anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibodies were from Transduction Laboratories. Anti-phospho-Tyr397 FAK and anti-phospho-Tyr418 Src antibodies were obtained from Calbiochem.

**Sources.** Anti-vinculin, anti-phospho-Ser AKT and anti-total AKT antibodies were purchased from Cell Signaling Technology Inc. Anti-EGFR monoclonal antibody, horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20, and anti-caspase-3 and anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibodies were from Transduction Laboratories. Anti-phospho T341/378 ERK1/2 antibodies were obtained from Amersham Biosciences. Anti-vinculin, anti-α-tubulin, and anti-β-actin antibodies were obtained from Sigma. Anti-hemagglutinin (HA) monoclonal antibody was from Roche Molecular Biochemicals. The tyrosphostin AG1478, an inhibitor of EGFR autophosphorylation, was obtained from Calbiochem. PD98059, an inhibitor of ERK1/2, was obtained from Calbiochem.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Polyclonal antibodies to p125 FAK (A17) and anti-ERK1/2 antibodies were obtained from Promega. Anti-phospho-Ser AKT and anti-total AKT antibodies were obtained from Cell Signaling Technology Inc. Anti-EGFR monoclonal antibody, horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20, and anti-caspase-3 and anti-poly(ADP-ribose) polymerase (PARP) monoclonal antibodies were from Transduction Laboratories.
check equal loading of proteins. Immunoblots were developed with Renaissance chemiluminescence reagent (PerkinElmer Life Sciences).

**Immunostaining**—Attached or detached suspended cells (collected by centrifugation and spread evenly on the slide) were fixed in 4% paraformaldehyde in 1× PBS for 10 min and permeabilized with 0.2% Triton X-100 for 5 min on ice. Cells were blocked with 25% normal goat serum in 1× PBS for 30 min, washed with 1× PBS, and incubated with primary antibody diluted 1:200 in 25% goat serum in 1× PBS. Cells were washed three times with 1× PBS, and a TRITC-conjugated secondary antibody (1:400 dilution in 25% goat serum) was applied to the coverslip. After washing three times with 1× PBS, cells were incubated with fluorescein isothiocyanate Bodipy-phallacidin (1:25 dilution in 25% goat serum; Molecular Probes, Inc.) for actin staining. For co-immunostaining experiments, cells were incubated with another primary antibody diluted 1:100 in 25% goat serum in 1× PBS for 1 h. After washing three times with 1× PBS, a Hoechst 33342 or spread evenly on a slide for TUNEL staining. In brief, after washing three times with 1× PBS, and a TRITC-conjugated secondary antibody (1:400 dilution in 25% goat serum) was applied to the coverslip. After washing three times with 1× PBS, and a TRITC-conjugated secondary antibody (1:400 dilution) was applied to the coverslip.

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

**RESULTS**

**EGF-dependent Tyrosine Phosphorylation and Its Inhibition by AG1478 in BT474-EGFR Cells**—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 with the pcDNA3 vector control cells (called BT474-pcDNA3 cells) (Fig. 1A). In the BT474-EGFR cells, treatment with EGF at 10 ng/ml rapidly increased EGFR tyrosine phosphorylation, and this effect was inhibited in the presence of the tyrosostatin 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 upon AG1478 treatment (Fig. 1D). In addition, AKT (Ser473) and FAK (phospho-Tyr397 and phospho-Tyr577) (data not shown) were highly phosphorylated in the 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. 2A, left panels). In these experiments, anti-FAK antibodies precipitated EGFR that was tyrosine-phosphorylated in the presence of EGF (Fig. 2A, upper left panel). Immunoprecipitation with anti-EGFR antibodies also precipitated FAK (Fig. 2A, right panels). The FAK and EGFR association was constitutive, as detected in the presence and absence of EGF (Fig. 2A). Thus, FAK and EGFR are physically associated in these breast cancer cells. Tyrosine phosphorylation of total cellular FAK was not affected by the EGFR inhibitor AG1478 (Fig. 2A, left panels), although a small portion of phosphorylated FAK that was associated with EGFR was inhibited by AG1478 (upper right panel). Dual immunofluorescence assays in individual cells demonstrated that FAK and EGFR were co-localized at focal adhesions in the BT474-EGFR cells (Fig. 2B, left panels) and also in the BT20 cells, which endogenously overexpress EGFR (right panels). These results show that FAK and EGFR associate and co-localize at the focal adhesions of these breast cancer cells.

**Overexpression of EGFR Suppresses Apoptosis Induced by FAK Down-regulation**—Next, we tested whether overexpression of EGFR protects breast cancer cells from detachment and apoptosis induced by FAK inhibition. In these experiments, we down-regulated FAK function using Ad-FAK-CD in the BT474-EGFR and BT474-pcDNA3 vector control cells. To inhibit FAK, we used an adenoviral construct of the C-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-lacZ as a control at an equal multiplicity of infection. These conditions resulted in >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 h of FAK-CD expression, there were similar levels of loss of adhesion in the vector control cells (93 ± 6%) and in the EGFR-overexpressing cells (83 ± 17%) (Fig. 4A) and only 0.5% detachment by control Ad-lacZ at the same dose (data not
Furthermore, inhibition of EGFR kinase activity with AG1478 did not enhance the loss of adhesion (Fig. 4A). These results show that overexpression of EGFR does not augment the ability of BT474 cells to resist loss of adhesion induced by inhibition of FAK function.

In contrast, EGFR suppressed apoptosis induced by FAK inhibition with Ad-FAK-CD. After 24 h of Ad-FAK-CD infection, detached BT474-EGFR cells had significantly reduced levels of apoptosis (66%) compared with the BT474-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 in the BT474-pcDNA3 control cells (Fig. 4B).

In Hoechst-stained cells (Fig. 4C), apoptotic condensed nuclei with fragmented chromatin in the 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 PD98059 (ERK1/2 inhibitor) in the BT474-EGFR cells, the level of apoptosis was increased to that in the 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 apoptosis-inducing effect caused by loss of FAK function.

**Fig. 2.** FAK constitutively associates with EGFR in BT474-EGFR cells. A. BT474 cells were stimulated with EGF as described in the legend to Fig. 1. Left panels, immunoprecipitation (IP) was performed with anti-FAK monoclonal antibody 4.47, and Western blotting (WB) 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 coprecipitated with EGFR. Right panels, the same experiment was performed with immunoprecipitation of EGFR, detecting FAK and EGFR association. The experiment was done three times with the same results. The images are composites from the same films/gels. B. FAK and EGFR association and co-localization at focal adhesions was detected by co-immunostaining. Immunostaining was performed with mouse anti-FAK monoclonal primary antibody 4.47 and probing with rhodamine-conjugated anti-mouse secondary antibody. After washing, dual immunostaining was performed with anti-EGFR monoclonal antibody and probing with fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody. The merged images were obtained with Adobe Photoshop Version 6.0.

**Fig. 3.** Adenovirus-mediated expression of FAK-CD and lacZ in BT474 cells. BT474 cells were infected with Ad (Adeno-lacZ and Ad-FAK-CD as described under “Experimental Procedures.” In titration experiments, the viral titer was detected with maximal cell infectivity. At the optimal viral concentration (500 focus-forming units/cell), ~100% of cells were X-gal-positive (blue) as 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 h of infection (B). B: upper panel, detached cells were stained with fluorescein isothiocyanate-Bodipy<sup>3</sup>-phallacidin, which stains actin inside cells, for better cell visualization of cells under a fluorescent microscope. Lower panel, HA-rhodamine immunostaining showed that 100% of the cells expressed HA-tagged FAK-CD. lacZ-positive cells were captured at ×40 magnification, and HA-tagged FAK-CD-expressing cells, which have a round morphology after 24 h, were captured at ×100 magnification under a fluorescent microscope.

shown). Furthermore, inhibition of EGFR kinase activity with AG1478 did not enhance the loss of adhesion (Fig. 4A). These results show that overexpression of EGFR does not augment the ability of BT474 cells to resist loss of adhesion induced by inhibition of FAK function.

In contrast, EGFR suppressed apoptosis induced by FAK inhibition with Ad-FAK-CD. After 24 h of Ad-FAK-CD infection, detached BT474-EGFR cells had significantly reduced levels of apoptosis (66%) compared with the BT474-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 in the BT474-pcDNA3 control cells (Fig. 4B). In Hoechst-stained cells (Fig. 4C), apoptotic condensed nuclei with fragmented chromatin in the 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 PD98059 (ERK1/2 inhibitor) in the BT474-EGFR cells, the level of apoptosis was increased to that in the 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 apoptosis-inducing effect caused by loss of FAK function.
FIG. 4. BT474 cells overexpressing EGFR suppress apoptosis, but not cell detachment induced by FAK down-regulation. A, Ad (Adeno)-FAK-CD infection stimulates cell detachment in BT474 cells. BT474-pcDNA3 and BT474-EGFR cells were infected with Ad-FAK-CD or Ad-luc2 (see “Experimental Procedures”) at 500 focus-forming units/cell, resulting in 100% cell infectivity. After 24 h, Ad-FAK-CD-infected detached cells were counted using a hemocytometer. Ad-luc2-infected cells were resistant to detachment and were not apoptotic (data not shown). Four independent experiments were done with the same results, and a representative experiment is shown. The mean percent ± S.D. of detached cells is shown from three independent cell counts using a hemocytometer. B, apoptosis induced by Ad-FAK-CD is inhibited by EGFR overexpression and reversed by the 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-luc2-infected cells were not apoptotic (data not shown). Bars represent mean values ± S.D. More than 100 cells were counted in 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 had bright Hoechst staining of condensed nuclear chromatin. Ad-luc2-infected cells did not undergo apoptosis and had unfragmented nuclei, as did control BT474 cells (data not shown). Bars represent mean values ± S.D. More than 100 cells were counted in three independent fields for each experimental treatment in three independent experiments. D, the ERK1/2 inhibitor PD98059 increases Ad-FAK-CD-induced apoptosis in BT474-EGFR cells. BT474-EGFR cells were pretreated with 10 μM PD98059 for 15 min before Ad-FAK-CD treatment. PD98059 was present during adenoviral incubation for 24 h. After 24 h of adenoviral infection, apoptosis was measured as described for B. The graph shows PD98059-increased apoptosis in BT474-EGFR cells. Bars represent means ± S.D. More than 100 cells were counted in three independent fields for each experimental treatment in three independent experiments. Statistical significance was determined using Student’s t test. *a significant difference from the pcDNA3 control (p < 0.02); **, a significant difference from the EGFR sample (p < 0.040). Hoechst-stained nuclei are also shown.
same results, and a representative experiment is shown. 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 was constitutively expressed and Ser\(^{\text{473}}\)-phosphorylated in the BT474-EGFR cells as well as in the BT474-pcDNA3 control cells treated with control Ad-lacZ (Fig. 5). The BT474-pcDNA3 cells treated with Ad-FAK-C5 down-regulated AKT, as total AKT and Ser\(^{\text{473}}\)-phosphorylated AKT were not present in these cells (Fig. 5, third and fourth panels, third lanes). In contrast to the BT474-pcDNA3 control cells, the BT474-EGFR cells (independent of EGF ligand) expressed AKT and the active Ser\(^{\text{473}}\)-phosphorylated form of AKT in response to down-regulation of FAK (Fig. 5). However, when EGFR kinase activity was inhibited by AG1478 in the BT474-EGFR cells, there were undetectable levels of AKT protein upon Western blotting, 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 down-regulation in response to FAK inhibition.

To further assess the relationship of EGFR overexpression and FAK down-regulation to the ERK1/2 and AKT pathways in BT474 cells, we inhibited TNF family signaling in the BT474-EGFR and BT474-pcDNA3 cells, based on our recent data that the TNFR family regulates FAK-CD-induced apoptosis (2). To analyze if these receptors are important in reduction of AKT protein levels in apoptotic BT474-pcDNA3 cells infected with Ad-FAK-C5, we blocked death receptor pathways by co-infection of cells with adenoviral dominant-negative FADD lacking amino acids 1–79 of the death effector domain (Ad-ΔFADD) (49). Under these conditions, where the death complex was inhibited, AKT protein levels were not reduced, and AKT was Ser\(^{\text{473}}\)-phosphorylated in the BT474-pcDNA3 and BT474-EGFR cells (Fig. 5, last two lanes). The results demonstrate that AKT down-regulation (reduction of protein levels) is mediated through a TNF family pathway in the BT474-pcDNA3 cells infected with Ad-FAK-C5. 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 TNFR family pathways (Fig. 5). These results show that TNFR family signaling, which is involved in FAK-CD-induced apoptosis, is also important in down-regulation/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 MAPK (ERK1/2) and TNFR family-dependent AKT signaling pathways.

EGFR Overexpression Protects p125\(^\text{FAK}\) and Caspase-3 and -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 endogenous p125\(^\text{FAK}\) in the breast cancer cells from degradation in response to Ad-FAK-C5. Previous work from our group has shown that p125\(^\text{FAK}\) is degraded 24 h after Ad-FAK-C5 infection in parental BT474 cells and that this effect is mediated through caspase-8 and -3 (2). Similarly, in these studies, the p125\(^\text{FAK}\) protein was degraded in the BT474-pcDNA3 control cells upon infection with Ad-FAK-C5 (Fig. 5). However, in the BT474-EGFR cells, EGFR overexpression protected p125\(^\text{FAK}\) from complete degradation by Ad-FAK-C5, as shown by the 125-kDa FAK band that was present in the BT474-EGFR cells, but not in the BT474-pcDNA3 cells (Fig. 5, third through fifth lanes). Down-regulation of both FAK and EGFR did lead to complete degradation of p125\(^\text{FAK}\) 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, sixth and seventh lanes). Co-infection of the BT474 cells with Ad-FAK-C5 and Ad-ΔFADD blocked cleavage of FAK (Fig. 5).

In the BT474-pcDNA3 cells, treatment with Ad-FAK-C5...
induced caspase-3 activation and PARP (caspase-3 substrate) cleavage (Fig. 6A). Pretreatment of these cells with the Ac-DEVD-CHO peptide (a caspase-3 family inhibitor) prior to infection with Ad-FAK-CD blocked activation of caspase-3 and cleavage of PARP and increased the levels 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 BT474 cells.

In contrast, overexpression of EGFR protected caspase-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, fourth and fifth lanes). However, when EGFR was inhibited under these conditions, the cleavage of caspase-3 and PARP was restored to levels equivalent to those in the BT474-pcDNA3 cells (Fig. 6B, third, sixth, and seventh lanes). 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 the BT474-pcDNA3 cells with Ad-FAK-CD and Ad-ΔFADD protected caspase-8 and -3 and PARP from cleavage (Fig. 6).

**Dual Inhibition of FAK and EGFR in BT20 Breast Cancer Cells, Which Endogenously Overexpress EGFR, Enhances Apoptosis, Down-regulating 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, which 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 BT20 breast cancer cells induced loss of adhesion, although this effect was seen at later time points, 46–71 h after adenoviral infection. The BT20 cells treated with Ad-FAK-CD started to detach at 46 h, and >60% of the cells treated with FAK-CD alone or with AG1478 in the absence or presence of EGF detached by 71 h (Fig. 7A).

However, inhibition of FAK and EGFR enhanced the levels of apoptosis in these cells (Fig. 7B) in a fashion similar to that seen in the BT474-EGFR cells (Fig. 4B). At 46 h after infection, the levels of apoptosis were slightly increased when cells were

![Fig. 6. Caspase-3-dependent degradation of FAK and AKT in BT474 cells.](image-url)
treated with FAK-CD and AG1478 compared with cells that had been treated with FAK-CD alone (7% versus 1.6%). However, at 71 h, 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% (without and with EGF, respectively) (Fig. 7B).

Next, we directly compared the biochemical effects of FAK and EGFR inhibition in the BT474-EGFR and BT20 cells. As shown by control Ad-lacZ infection, the levels of endogenous EGFR and p125\textsuperscript{FAK} expression as well as the levels of AKT phosphorylation were higher in the BT20 cells (Fig. 8A, sixth lane) than in the BT474-pcDNA3 cells (first lane) or the BT474-EGFR cells (second lane). After 24 h of infection with Ad-FAK-CD, the BT20 cells totally protected FAK from degradation detected in the BT474-pcDNA3 cells (Fig. 8A). The ERK1/2 survival pathway was activated in the BT20 cells (as in the BT474-EGFR cells), as ERK1/2 was highly phosphorylated in the BT474-EGFR cells than in the BT474-pcDNA3 cells (Fig. 7B). The combination of AG1478 and show that dual inhibition of EGFR and FAK down-regulation in the AG1478- and Ad-FAK-CD-treated BT20 cells was caspase-3-dependent. These results mechanistically support the increased levels of apoptosis seen upon 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 the BT20 cell line, inhibiting the same survival signaling pathways as the BT474-EGFR cells.

**DISCUSSION**

This study demonstrates the cooperativity of both FAK and EGFR signals in suppressing apoptosis in breast cancer cells. Although 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 at a biochemical level, whereby the EGFR-overexpressing cells had increased levels of both ERK1/2 and AKT phosphorylation and did not demonstrate complete p125\textsuperscript{FAK} or caspase-3 or -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 (Fig. 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\textsuperscript{−/−} fibroblasts in the presence of EGF (27), suggesting that FAK can mediate a link between growth factor receptors and integrins. 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 (22), providing further evidence that FAK can integrate motility signals from EGF to EGFR. Other motility studies suggest that FAK is dephosphorylated in response to EGF, promoting tumor invasion and motility (28). 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 breast cancer cells in our system and found that...
In breast cancer cells are not simply limited to motility and invasion, but can cooperate with EGFR signaling to suppress apoptosis and to enhance survival of breast cancer cells. Studies of FAK in primary breast cancer specimens have shown that up-regulation of FAK expression is an early event in tumorigenesis, occurring in ductal carcinoma 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 tumors and the matrix and the other providing survival signals to resist apoptosis (2). Our results in this study also support this hypothesis, whereby down-regulation of FAK function had effects on apoptosis in EGFR-overexpressing breast cancer cells.

In this study, 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 down-regulation. This is consistent with other studies implicating the ERK pathways in apoptotic resistance, including tumor necrosis factor-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 was also associated with the inability of FAK inhibition to cause down-regulation and dephosphorylation of AKT in EGFR-overexpressing cell lines. This suggests that EGFR also has a survival signal function through this serine/threonine kinase. Furthermore, this effect appears 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 phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58). In a model of oxidative stress-induced apoptosis, H2O2 activates AKT via phosphatidylinositol 3-kinase (42, 56–58).
These observations have recently been extended to TRAIL-induced apoptosis, whereby signaling from EGFR to AKT protects HEK293 and MDA-MB-231 cells from apoptosis by inhibiting mitochondrial cytochrome c release (57). In our system, the apoptosis induced by FAK down-regulation 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, inhibition of the death complex with dominant-negative FADD, consistent with our previous studies (2). In addition, these observations have recently been extended to TRAIL-induced apoptosis, whereby signaling from EGFR to AKT protects HEK293 and MDA-MB-231 cells from apoptosis by inhibiting mitochondrial cytochrome c release (57). In our system, the apoptosis induced by FAK down-regulation 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.

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