Chemotherapeutic drugs that damage DNA kill tumor cells, in part, by inducing the expression of a death receptor such as Fas or its ligand, FasL. Here, we demonstrate that epidermal growth factor (EGF) stimulation of T47D breast adenocarcinoma and embryonic kidney epithelial (HEK293) cells protects these cells from Fas-induced apoptosis. EGF stimulation of epithelial cells also inhibited Fas-induced caspase activation and the proteolysis of signaling proteins downstream of the EGF receptor, Cbl and Akt/protein kinase B (Akt). EGF stimulation of Akt kinase activity blocked Fas-induced apoptosis. Expression of activated Akt in MCF-7 breast adenocarcinoma cells was sufficient to block Fas-mediated apoptosis. Inhibition of EGF-stimulated extracellular signal-regulated kinase (ERK) activity did not affect EGF protection from Fas-mediated apoptosis. The findings indicate that EGF receptor stimulation of epithelial cells has a significant survival function against death receptor-induced apoptosis mediated by Akt.

Expression of epidermal growth factor (EGF) receptor family members (ErbB1–4) is observed in many human tumors, particularly of the breast, ovary, and lung (1–4). Overexpression of EGF receptors in these tumors is correlated with poor prognosis for responsiveness to radiation and chemotherapy (1, 5–7). The proliferative response and resistance to chemotherapy afforded by EGF receptor activation in these tumors may allow for the rapid outgrowth of drug-resistant tumor cells (3, 6). Clinically, there is interest in developing strategies to test if expression of Fas and/or FasL (12). Blocking Fas activation in these cells inhibits drug-induced apoptosis of the tumor cells (12, 13, 15). These findings have demonstrated a signal transduction response from DNA damage to the transcriptional regulation of death receptors and their ligands. In this report we show that activation of EGFR receptors in tumor cells of epithelial origin protects against Fas-induced apoptosis.

MATERIALS AND METHODS

Cell Culture—Cells were maintained in a humidified 7.0% CO2 environment in Dulbecco’s modified medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Inc.). Media for T47D, MCF7, and HEK293 cells was supplemented with 20% bovine calf serum, 10% fetal bovine serum, and 10% bovine calf serum (Life Technologies, Inc., respectively). MCF7 cells expressing vector Akt K-M and Akt-myr protein were under selection with 0.5 mg/ml G418 (Life Technologies, Inc.). Each cell line was tested for the presence of EGF receptors and the Fas receptor.

EGF and Fas Stimulation—Cells (1–2 x 106) were suspended in 1 ml of Dulbecco’s modified medium containing serum to minimize incubation volumes. The cells were incubated with or without 1 μg/ml EGF (Biochemical Technology) for 1 h at 37 °C. After the incubation, 1 μg/ml activating anti-Fas antibody (Upstate Biotechnology Inc.) was added, and cells were incubated at room temperature for 10 min. Two ml of Dulbecco’s modified medium-containing serum was added to the cells, and the mixture was placed in tissue culture plates. Adherent cells were incubated at 37 °C in a 7.0% CO2 incubator for 24 or 48 h where indicated. Wortmannin (100 μM, Sigma) and PD098059 (50 μM, Parke-Davis) was added at the same time as EGF where indicated. Similar results were obtained when EGF and activating anti-Fas antibody were added directly to adherent cells.

Akt Kinase Assay—Cells were lysed in Akt lysis buffer (10 mM K2PO4, pH 7.4, 1 mM EDTA, pH 8.0, 5 mM EGTA, 10 mM MgCl2, 20 mM β-glycerophosphate, 0.5 mM sodium vanadate, 2 mM diethiothreitol, 40 mg/ml phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40, 40 μg/ml aprotinin, and 40 μg/ml leupeptin). Cellular debris was removed by centrifugation at 8,000 x g for 5 min. Protein concentration was determined by a Bradford assay using bovine serum albumin as a standard. Four hundred μg of cell lysates was immunoprecipitated with 2 μg/ml of an anti-Akt1 antibody (Santa Cruz Biotechnology Inc.) for 1 h at 4 °C with agitation. This was followed by the addition of 15 μl of a 1:1 slurry of protein A-Sepharose beads (Sigma) and incubated at 4 °C for 1 h. The
beads were then washed twice in 1 ml of lysis buffer and twice in Akt wash buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 1 μg/ml protein kinase A inhibitor peptide). Thirty-five μl of the last wash was left in the tube and mixed with 25 μl of Akt reaction mix (Akt wash buffer, 0.2 mM ATP, 0.2 μg/ml cross-tide peptide (GRPRTSSFAEG), 0.2 μCi/μl [γ³²P]ATP) and incubated for 20 min at 30 °C. The reaction was stopped with 10 μl of 0.5 M EDTA and spotted on P81 Whatman paper. The samples were washed three times for 5 min each in 75 mM phosphoric acid, air-dried, and counted in a β counter.

ERK Kinase Assay—Cells were lysed in TX-100 lysis buffer (70 mM β-glycerophosphate, 1 mM EGTA, 100 μM sodium vanadate, 1 mM dithiothreitol, 2 mM MgCl₂, 0.5% Triton X-100, 20 μg/ml aprotinin). Lysate were treated as described in Akt kinase assay. ERK2 was incubated with 2 μg/ml anti-ERK2 antibody (Santa Cruz Biotechnology Inc.) for 1 h as described for the Akt kinase assay. The beads were washed twice with 1 ml of lysis buffer and twice with 1 ml of lysis buffer without Triton X-100. Thirty-five μl of the last wash was left in the tube and mixed with 20 μl of ERK reaction mix (50 mM β-glycerophosphate, 100 μM sodium vanadate, 20 mM MgCl₂, 200 μM ATP, 0.5 μCi/μl [γ³²P]ATP, 400 μM epidermal growth factor receptor peptide 662–681, 100 μg/μl IP-20, 2 mM EGTA) incubated for 20 min at 30 °C. The reaction was stopped with 10 μl of 500 mM EDTA and spotted on to P81 Whatman paper. The samples were washed four times with 75 mM phosphoric acid, air-dried, and counted in a β counter.

Immunoblots—Cells were lysed in Nonidet P-40 lysis buffer (50 mM HEPES, pH 7.25, 150 mM NaCl, 50 mM ZnCl₂, 50 mM NaF, 2 mM EDTA, 1 mM sodium vanadate, 1.0% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride). Cell debris was removed by centrifugation at 8,000 × g for 5 min, and protein concentration was determined by a Bradford assay. Two hundred to 400 μg of cell lysate protein was subject to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline, 5% milk. Blots were performed as described in Widmann et al. (19).

Measurement of Apoptosis—Cells (1–2 × 10⁶) were resuspended in...
a coverslip was applied over the 10 μm. The blots were stripped and reprobed with an anti-Akt antibody. The results are representative of three independent experiments. Incubated for 24 h with or without 1 μg/ml EGF and with or without 1 μg/ml activating anti-Fas antibody. As indicated, cells were stimulated an additional 5 min with 1 μg/ml EGF. The cells were lysed and Western blotted using an antibody directed against the phosphoserine 473 of Akt. The blots were stripped and reprobed with an anti-Akt antibody. The results are representative of three independent experiments.

100 μl of media by gentle vortexing and 2 μl of acridine orange (100 μg/ml) and 100 μg/ml ethidium bromide in phosphate-buffered saline was added. Ten μl was removed and placed on a microscope slide, and a coverslip was applied over the 10 μl. The slide was viewed on a fluorescence microscope using a fluorescein filter set for the detection of condensed DNA in apoptotic cells. The condensed DNA was determined by intense local staining of DNA in the nucleus compared with the diffuse staining of the DNA in normal cells. The percentage of apoptotic cells was determined from cells containing normal DNA staining compared with cells with condensed nuclei. Apoptosis was verified by propidium iodide staining for DNA fragmentation and morphological changes consistent with apoptotic cells.

RESULTS

EGF Stimulation of Epithelial Cells Inhibits Fas-induced Apoptosis—EGF stimulation of T47D breast adenocarcinoma and human embryonic kidney epithelial (HEK293) cells significantly diminished the percentage of apoptotic cells in response to activation of Fas relative to control cells not exposed to EGF (Fig. 1A). Stimulation of Fas in T47D cells resulted in 57 and 74% of the cells to be apoptotic at 24 and 48 h, respectively. Forty-eight h after activation of Fas in HEK293 cells, 66% of the cell population was apoptotic. EGF stimulation before Fas ligation reduced the apoptotic response 48 h later to 38% and 32% in T47D and HEK293 cells, respectively. Similar results were observed with activation of Fas by an activating anti-Fas antibody (Fig. 1A) or soluble FasL (not shown). Quantitation of apoptosis by acridine orange or propidium iodide staining gave similar results. The findings show that in two different epithelial cell types, EGF stimulation reduces the ability of Fas activation to induce cell death by apoptosis.

Fas-mediated Caspase Activation Is Inhibited by EGF Stimulation of Epithelial Cells—Caspases are the cysteine proteases that when activated cleave proteins at defined aspartic acid-containing recognition sequence motifs (16, 17). The cumulative action resulting from caspase cleavage events commit cells to apoptosis (16–18). Measurement of caspase activity using a fluorescent DEVD peptide substrate for caspase 3-like proteases demonstrated Fas stimulates caspase activity 3- and 2-fold, respectively, in T47D and HEK293 cells (Fig. 1B). Prior treatment of T47D and HEK293 cells with EGF effectively inhibits caspase activation in response to Fas ligation in both cell types. This result indicates that EGF stimulates signal pathways that inhibit Fas activation of caspases. The decreased caspase activation would effectively inhibit Fas-mediated apoptosis.

We recently demonstrated that Fas stimulated caspase-dependent cleavage of several proteins involved in EGF signaling including Akt and Cbl (19). Consistent with the inhibition of caspase activation by EGF (Fig. 1B), EGF stimulation of T47D and HEK293 cells inhibited the proteolysis of Akt and Cbl in response to Fas ligation (Fig. 1C). Protection of Cbl and Akt from caspase-catalyzed cleavage was maintained even after 48 h of Fas activation in the continued presence of EGF (not shown). The expression of mitogen-activated protein kinase kinase kinase 1 (MEK1), which is not a caspase substrate (19), is not changed during Fas activation in cells. Thus, the cleavage by caspases of specific signaling proteins, but not every signaling protein, is orchestrated during the apoptotic response initiated by Fas ligation. EGF stimulation of T47D and HEK293 cells inhibits the caspase-dependent cleavage of proteins in response to ligation of the death receptor, Fas.

Activation of Akt Protects It from Fas-induced Degradation—Akt is activated by EGF stimulation in several cell types (20, 21). Indeed, EGF stimulation of T47D cells activates Akt (Fig. 2A). Akt activation also has been shown to protect cells from stress-induced apoptosis (22–24). The caspase-dependent cleavage of Akt would be expected to prevent this anti-apoptotic response. Therefore, we examined if EGF stimulation of T47D cells sustains Akt activation in Fas-activated cells. The activation of Akt was determined with an antibody specific for the phosphorylated serine 473 of Akt. Serine 473 is located in the kinase domain of Akt, and its phosphorylation is required to indicate Akt activation (23, 24). Increased phosphorylation of Akt was detected within 5 min after EGF stimulation (Fig. 2B). Twenty-four h after Fas ligation, Akt was still activated in EGF-treated cells (Fig. 2B). Furthermore, a second challenge with EGF increased the phosphorylated activated form of Akt in cells exposed to Fas. In contrast, Fas activation in cells that were not prestimulated with EGF resulted in no measurable Akt protein (Fig. 2B). These results show that EGF stimulation of T47D cells not only activates Akt but also protects it from degradation by caspases in Fas-stimulated cells.

Akt but Not ERK Activation Is Required for EGF-mediated Protection from Apoptosis—The ability of specific signal transduction pathways to have anti-apoptotic regulatory properties is variable in different cell types (25). For example, both Akt and the ERK mitogen-activated protein kinase pathway have been shown to be anti-apoptotic in different cell types and in response to different stress stimuli (23, 25–28). Fig. 3 shows
that EGF stimulation of T47D cells activates both Akt and ERK activities. The time course in response to EGF stimulation of ERK activation (Fig. 3A) is similar to that for Akt (Fig. 2A), although ERK activation peaked at 10 min, whereas Akt activation peaked at 30 min. Activation of ERK in response to EGF stimulation is effectively inhibited by incubation of T47D cells with the Parke-Davis compound, PD098059 (Fig. 3B). This compound stimulates the degradation of MEK1 and -2, the mitogen-activated protein kinase kinase in the ERK pathway (29). The activation of Akt is regulated by phosphorylated phosphatidylinositols that are formed as products of phosphatidylinositol 3-kinase-catalyzed reactions. The pleckstrin homology domain of Akt binds to the plasma membrane-associated phosphatidylinositol (3, 4, 5) trisphosphate or phosphatidylinositol (3, 4) bisphosphate, resulting in the translocation of Akt from the cytosol to the plasma membrane. Membrane-associated Akt is phosphorylated by phosphoinositide-dependent kinase, leading to its activation (20, 22). As predicted, inhibition of phosphatidylinositol 3-kinase by wortmannin inhibits EGF-stimulated Akt activity in T47D cells (20, 30, 31) (Fig. 3C).

Fig. 4 shows that wortmannin but not PD098059 inhibits the ability of EGF to protect T47D cells from Fas-induced apoptosis. The anti-apoptotic function of EGF is completely abrogated by pretreatment with wortmannin (Fig. 4A). In contrast, inhibition of ERK activation has no effect on the ability of EGF to protect against Fas-induced apoptosis. The ability of wortmannin to inhibit the anti-apoptotic function of EGF is mirrored in the regulation of caspase activity (Fig. 4B). The suppression of Fas-induced caspase activity by EGF stimulation of T47D cells is completely inhibited by wortmannin treatment (Fig. 4B). The effect of wortmannin on caspase activation is also demonstrated by wortmannin blocking the ability of EGF to protect Cbl and Akt from caspase-mediated degradation (Fig. 4C).

The ability of Akt to protect epithelial cells from Fas-induced apoptosis is also observed in MCF-7 breast adenocarcinoma cells (Fig. 5). As shown in T47D cells, EGF stimulation suppresses Fas-induced apoptosis (Fig. 5A). Stable expression of a constitutively activated Akt in MCF-7 cells lowers the basal
apoptotic cell index relative to control MCF-7 cells (Fig. 5B). In contrast, a kinase-inactive form of Akt (Akt K-M) has no effect on the basal apoptotic index of MCF-7 cells. Stimulation of Fas results in a significant apoptotic response in both Akt K-M and control MCF-7 cells. In contrast, expression of the myristoylated constitutively active form of Akt (Akt-myr) strongly protected MCF-7 cells from Fas-induced apoptosis. Thus, activation of Akt seems sufficient to inhibit Fas-induced apoptosis.

**DISCUSSION**

We have shown that EGF stimulation of three different cell lines protects epithelial cells from Fas-induced apoptosis. The activation of Akt appears to be both required and sufficient for the anti-apoptotic function of EGF (Fig. 6). EGF also has been shown to protect rat prostatic epithelial cells and cultured fetal hepatocytes from tumor necrosis factor β-induced apoptosis (32, 33). In other cell types the potential anti-apoptotic function of EGF is less apparent. In fibroblasts, EGF is not able to significantly protect against UV-B-induced apoptosis unless highly overexpressed (21, 34). In contrast, insulin-like growth factor 1 was able to block UV-B-induced apoptosis (21). Similarly, EGF was not able to block tumor necrosis factor α-induced apoptosis in adipocytes (35). The differential ability of EGF to regulate Akt activity in these cell types may explain these differences. Thus, it appears that epithelial cells and fibroblasts have different abilities for EGF to signal anti-apoptotic responses. It is not clear whether this is because of receptor concentration or which EGF receptor family members are present.

It should be noted that even though the ERK pathway did not have an anti-apoptotic function in the three epithelial cell lines we tested, ERK has been shown to have significant survival functions in other cell types. For example, in L929 fibrosarcoma cells and human neutrophils, the ERK pathway prevents tumor necrosis factor α and UV-induced apoptosis, respectively (36–38). Thus, different cell types may use either Akt or ERK signaling pathways for survival in response to different pro-apoptotic stimuli. Recently, it was shown that Akt phosphorylates both Bad and caspase 9, inhibiting their pro-apoptotic activity. The phosphorylation of these proteins appears to be a mechanism for the anti-apoptotic function for Akt (18, 39, 40). The mechanism by which ERK can function to protect specific cell types from apoptosis is not currently defined.

Our finding that in human breast epithelial tumor cells, EGF protects against apoptosis is particularly relevant to understanding the function of EGF receptors in breast cancer. Human breast tumor cells express EGF receptors that contribute to their growth and survival (1, 3, 6). Many breast cancer cells express multiple members of the ErbB family including ErbB1 (EGF receptor) and ErbB2 (Neu) (1, 6). In addition, many breast cancers also express either EGF or transforming growth factor α and are thus able to activate EGF receptors in the tumor cells via autocrine and paracrine mechanisms (41). The stimulation of the Akt-signaling pathway in breast tumors would also be predicted to provide a survival function during the transformation process. Overexpression of ErbB2 receptor by transfection of MDA-MB-435 breast cancer cells resulted in protection of these cells from taxol-induced apoptosis, indicating that EGF receptor stimulation can also confer drug resistance (42). Our results indicate that EGF stimulation of breast
Akt inhibits Fas-induced apoptosis

The finding that EGF protects breast cancer cells from apoptosis is consistent with the recent observations using the anti-Neu (ErbB2) antibody, Herceptin (4). Herceptin blocks EGF signaling and induces apoptosis of breast cancer cells and is now in use clinically in the treatment of breast cancer. It is possible that inhibition of the Akt stimulatory pathway will further sensitize cells to Herceptin-induced apoptosis (4). Combined with chemotherapy, multimodal treatment would effectively induce apoptosis in part by making the tumor cells more sensitive to Fas activation. The ability to define the anti-apoptotic potential of signaling pathways like that for Akt in different tumor types should allow enhanced efficiency of treatments involving drugs like Herceptin, DNA damaging drugs, and microtubule toxins that induce the apoptosis of human tumors. Future studies will evaluate the ability of EGF to protect cells against chemotherapeutic drugs in vitro and in xenograft tumor models in nude mice.

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