Epidermal Growth Factor Protects Epithelial-derived Cells from Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis by Inhibiting Cytochrome c Release

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in combination with chemotherapeutic drugs induces a synergistic apoptotic response in cancer cells. TRAIL death receptors have also been implicated in chemotherapeutic drug-induced apoptosis. This has lead to TRAIL being proposed as a potential cancer treatment. In nude mice injected with human tumors, TRAIL reduces the size of these tumors without toxic side effects. We demonstrate that the epidermal growth factor (EGF) stimulation of human embryonic kidney cells HEK 293 and breast cancer cell line MDA MB 231 effectively protects these cells from TRAIL-induced apoptosis in a dose-dependent manner. This stimulation blocks apoptosis by inhibiting TRAIL-mediated cytochrome c release from the mitochondria and caspase 3-like activation. EGF survival response involves the activation of AKT. Expression of activated AKT was sufficient to block TRAIL-induced apoptosis, and kinase-inactive AKT expression blocked EGF-protective response. In contrast, inhibition of EGF stimulation of extracellular-regulated kinase (ERK) activity did not affect EGF protection. These findings indicate that EGF receptor activation provides a survival response against TRAIL-induced apoptosis by inhibiting mitochondrial cytochrome c release that is mediated by AKT activation in epithelial-derived cells.

INTRODUCTION

EGF and its receptors are expressed in many cancers including breast, ovary, and lung (1, 2). Overexpression of EGF receptors is correlated to an aggressive form of cancer leading to poor prognosis (2, 3). The poor prognosis might be attributable to decreased responsiveness to chemotherapy and radiation treatments (4). The ability of EGF receptor activation to increase proliferative responses and induce resistance to chemotherapy may allow for the rapid outgrowth of drug-resistant tumor cells. Thus, it is important to understand how EGF contributes to tumor tolerance of chemotherapeutic drugs and develop new strategies to overcome this resistance.

Administration of a member of the death receptor (DR) ligand family, TRAIL, to nude mice containing human tumors ranging from breast to lung tumors, reduced the size of these tumors (5). It was also demonstrated that TRAIL fails to induce apoptosis in normal surrounding tissues in mice and in human primary tissue culture cells but is effective at inducing apoptosis in cancer cell lines (5). TRAIL was also nontoxic to mice, whereas DR ligands TNF and FAS ligand showed toxic side effects (5). Treatment of a wide variety of different cancer cells with TRAIL, and chemotherapeutic drugs causes a synergistic apoptotic response (5, 6). This is unique to TRAIL, because other DR ligands fail to show this effect in combination with chemotherapeutic drugs. This synergistic apoptotic response is at least partially attributable to chemotherapeutic drug-mediated increase expression of the TRAIL DRs, DR4 and DR5 (7). Thus, TRAIL alone or in combination with chemotherapeutic drugs could be used as a molecular-based treatment for cancer where other DR ligands have failed.

TRAIL induces apoptosis through activation of its DRs, DR4 and DR5 (8). Treatment with the chemotherapeutic drugs etoposide and doxorubicin increases the expression of DR4 and DR5, as well as other DRs (7, 9, 10). Blocking DR activation by either sequestering the ligand away from its DR or by using antibodies against the DR thereby blocking binding of its ligand inhibited etoposide- and doxorubicin-induced apoptosis (7, 9, 10). DRs induce apoptosis through the binding of the adaptor protein FADD to their death domains. Caspase 8, which is constitutively associated with FADD, is activated by FADD recruitment to DRs. This activation causes the cleavage of specific intracellular proteins leading to the induction of apoptosis (8). The BCL2 family member BID is cleaved by caspase 8. On cleavage, truncated BID translocates to the mitochondria contributing to the release of cytochrome c (11, 12). This release of cytochrome c leads to caspase 3-like activation causing apoptosis.

Treatment of epithelial cells with EGF has been shown to protect against FAS-induced apoptosis, whereas ErbB2/neu overexpression protects against apoptosis by the chemotherapeutic drug Taxol (13–15). Blocking the activation of EGF family receptors, using the monoclonal antibodies against EGF receptor (C225) or ErbB2/neu (Herceptin) exhibits antitumor effects in breast cancer (16–18). Thus, the presence of EGF and its receptors can contribute to resistance against apoptosis. Because TRAIL gives a synergistic apoptotic response in combination with chemotherapeutic drugs and is nontoxic to both mice and nonhuman primates, it is proposed as an effective treatment for cancer. Herein we show that treatment of epithelial-derived cells with EGF prevents TRAIL-induced apoptosis and caspase 3-like activation by inhibiting cytochrome c release from the mitochondria suggesting that EGF could affect the ability of TRAIL to treat epithelial-derived tumors.

MATERIALS AND METHODS

Cell Culture. Cells were maintained in a humidified 5.0% CO2, 37°C incubator in DMEM supplemented with 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Medium for HEK 293 and MDA MB 231 cells was supplemented with 10% FBS (Life Technologies, Inc.). Cells expressing vector alone, AKT KM, and myr-AKT proteins were under selection with 1.5 mg/ml G418 (Life Technologies, Inc.). Pictures of cells were captured using an Olympus inverted microscope equipped with a Spot digital camera.

Isolation of his-tagged Soluble TRAIL. Escherichia coli (BL21), containing the pET plasmid with 101–281 a.a.-soluble TRAIL cDNA, was grown overnight under selection (50 μg/ml ampicillin) and diluted 1 in 2. The bacteria were treated with 1 mg of isopropyl-1-thio-β-D-galactopyranoside for 2 h and lysed in lysis buffer [10 mM Tris (pH 8.0), 100 mM NaCl, 0.1% NP40, 5 mM imidazole, and 2 mM PMSF]. The lysate was sonicated 4 × 15 s and...
centrifuged at 8000 × g for 30 min. Nickel Sepharose beads (500 µl) were added to the lysate and incubated for 2 h at 4°C. The beads were then washed in wash buffer [10 mM Tris (pH 8.0), 100 mM NaCl, 0.1% NP-40, and 5 mM Imidazole] three times and eluted with elution buffer (10 mM TRIS pH 8.0, 100 mM NaCl, 0.1% NP40, and 100–300 mM imidazole). The elutions were loaded on a SDS-polyacrylamide gel and stained with Coomassie Blue to visualize soluble TRAIL. The isolated TRAIL was used to treat cells in medium where indicated.

**EGF and TRAIL Stimulation.** EGF (1 µg/ml) was added to cells (1–2 × 10⁶) in culture medium for 1 h. After 1 h, 1 µg/ml of his-soluble TRAIL was added to the cells in the presence of EGF and incubated for the indicated times at 37°C. Wortmannin (100 nM; Sigma Chemical Co.) or PD98059 (50 µM; Parke-Davis) was added at the same time as EGF and TRAIL where indicated.

**Immunobots.** Cells were lysed in NP40 lysis buffer [50 mM HEPES (pH 7.25), 150 mM NaCl, 50 µM ZnCl₂, 50 µM NaF, 2 mM EDTA, 1 mM Na vanadate, 1.0% NP40, and 2 mM PMSF]. Cell debris was removed by centrifugation at 8000 × g for 5 min, and protein concentration was determined by a Bradford assay. Cell lysate protein (200–400 µg) was subject to SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline and 5% milk. Blots were incubated with the appropriate antibody concentration overnight (anticaspase 8 and anti-PARP were purchased from Upstate Biotechnology; anti-MKP1 was purchased from Santa Cruz Biotechnology Inc.; anti-phospho BAD and anti-BAD were purchased from New England Biolabs; and anti-Bid was a kind gift from Dr. Greenberg, Manitoba Institute of Cell Biology, Winnipeg, Manitoba, Canada). Washed three times in 1 × Tris-buffered saline, and incubated 1 h with the appropriate secondary antibody conjugated with alkaline phosphatase. Blots were visualized on X-ray film with enhanced chemiluminescence reagents (NEN DuPont).

**Caspase Assay.** Cells (1–2 × 10⁶) were treated with EGF and TRAIL as described previously. The caspase inhibitor Ac-DEVD-fmk (50 µM) was added to cells treated with TRAIL as a negative control. The cells were then lysed in caspase lysis buffer [10 mM HEPES (pH 7.4), 2 mM EDTA, 1 mM DTT, 1% NP40, 2 mg/ml aprotinin, 10 µM leupeptin, 10 µM pepstatin A, and 200 µM PMSF] and centrifuged at 8000 × g for 5 min. Protein (100 µg) was added to caspase reaction buffer [100 mM HEPES (pH 7.4), 20% glycerol, 5 mM DTT, and 0.5 mM EDTA] and 100 µM of the peptide Ac-DEVD-pNA (Biomol Plymouth Meeting, PA). This was incubated at 37°C in the dark for 4 h and read at 405-nm wavelength on a spectrophotometer. Reaction buffer and peptide without lysate was used as a control.

**Measurement of Apoptosis.** Cells (1–2 × 10⁶) were resuspended in 100 µl of medium by gently vortexing, and 2 µl of acridine orange (100 µg/ml) and ethidium bromide (100 µg/ml) in PBS was added. Ten µl was removed and placed on a microscope slide, and a coverslip was applied over the cells. 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 the DNA in nuclei 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 DNA fragmentation and morphological changes consistent with apoptotic cells. For detection of sub-G₁ peak by flow cytometry, cells were resuspended in 1 ml of 1 × PBS and 4 ml of 70% ice-cold ethanol. The cells were incubated overnight at 4°C and then washed in 1 × PBS. The cells were then resuspended in 250 µl of PI solution (50 µg/ml PI in 1× PBS) and 1 µl of 20 µg/ml RNase. The cells were incubated in the dark for 15 min at room temperature and then analyzed on a flow cytometer.

**Assessment of Cytochrome c Release.** HEK 293 cells were grown on glass coverslips and were treated with EGF alone, TRAIL alone or in combination for 24 h. The coverslips were collected and fixed in 3.7% formaldehyde in 1× PBS. They were then washed twice for five min with 500 µl of 1× PBS and 0.1% NP40. Mouse anticytochrome c at a dilution of 1:500 in 10% PBS was incubated with the coverslips for 1.5 h with gentle shaking. The coverslips were washed twice in 500 µl of 1× PBS and 0.1% NP40. Secondary antibody antikytochrome c3 (Chemicon) at 1:5,000 dilution in 10% PBS, 1× PBS, and 0.1% NP40 was incubated with the coverslip for 1.5 h with gentle shaking in the dark. The secondary antibody was removed, and the coverslips were incubated for six min in 1:2000 dilution of 20 nm of Hoescht stain (Sigma Chemical Co.) in 1× PBS and 0.1% NP40 in the dark. Coverslips were mounted onto slides with 4 µl of Fluoroguard antifade reagent (Bio-Rad). No fewer than 200 cells were scored per sample. Fluorescence was visualized and captured using a Zeiss Axiphot microscope equipped with a cooled charged-coupled device camera. For biochemical analysis of cytochrome c release, transfected HEK 293 cells were resuspended in CFS buffer [10 mM HEPES (pH 7.4), 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 0.1 mM PMSF, and 1 mM DTT] and dounce homogenized (50 strokes). The cells were spun down at 3000 rpm for 5 min at 4°C. The supernatant was then centrifuged for 15 min at 12,800 rpm at 4°C. The pellet was resuspended in 30 µl of H buffer (300 mM sucrose, 5 mM N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 200 µM EGTA) and represents the heavy membrane fraction. The supernatant was spun down at 41,000 rpm for 1 h and represents the cytosolic fraction. The fractions were loaded onto SDS-polyacrylamide electrophoresis and Western blotted for cytochrome c (anticytochrome c; Santa Cruz Biotechnology).

**RESULTS**

**EGF Treatment Prevents TRAIL-induced Apoptosis and Caspase Activation in HEK 293 Cells and MDA MB 231 Cells.** Human embryonic kidney (HEK 293), and breast cancer (MDA MB 231 and MCF-7) cells were stimulated with TRAIL (1 µg/ml) in the presence or absence of EGF (1 µg/ml). There was no significant amount of apoptosis as measured by DNA condensation over the first 16 h of incubation in HEK 293, MDA MB 231, and MCF-7 cells (Fig. 1A). At both 24 h and 48 h, TRAIL-induced apoptosis was significantly inhibited by EGF treatment (Fig. 1A). Specifically, TRAIL-induced apoptosis in 40% ± 9.2 of HEK 293 cells, 72% ± 7.2 of MDA MB 231 cells, and 36% ± 3.6 of MCF-7 cells after 48 h. In the presence of EGF, the amount of TRAIL-induced apoptosis was significantly reduced to 23% ± 1.6 in HEK 293 cells, 40% ± 8.3 in MDA MB 231 cells, and 16% ± 1.1 in MCF-7 cells after 48 h of treatment. Twenty-four h after EGF alone, TRAIL alone, or EGF and TRAIL treatment, control and EGF treated MDA MB 231 cells were attached to the tissue culture plates and appear healthy (Fig. 1B). Cells treated with TRAIL were rounded and no longer attached to the plate. The cells also showed blebbing and a reduction in size. These morphological changes are indicative of cells undergoing apoptosis. In cells treated with both EGF and TRAIL, many of the cells were still attached to the plate and the cells that were not attached appear to have less blebbing compared with cells treated with TRAIL alone, suggesting EGF protects cells against TRAIL-induced apoptosis. In addition to measuring DNA condensation and morphological changes, cells were stained with PI and analyzed on a flow cytometer for formation of a sub-G₁ peak indicating cells undergoing apoptosis. After 48 h of TRAIL treatment, 18% of HEK293 cells showed sub-G₁ peak formation (Fig. 1C). After pretreatment with EGF, the amount of sub-G₁ peak reduced to 1.8%. EGF alone showed similar results as untreated cells (data not shown). The total amount of apoptosis as determined by DNA condensation was higher than sub-G₁ peak analysis. This could be attributable to the sensitivity of the different assays. For example, the background level of apoptosis was lower in PI-treated cells as compared with cells stained with acridine orange. However, the degree of protection provided by EGF treatment was similar in both assays. Finally in HEK293 and MDA MB 231 cells, concentration of EGF needed to provide protection against TRAIL-induced apoptosis was determined. At low doses of EGF (20 ng/ml), there was protection of MDA MB 231 cells (26% ± 4.8) but little protection of HEK 293 cells (12% ± 7.8). As the doses of EGF increased from 200 ng/ml to 1 µg/ml, the amount of protection increased to a maximum of 40% ± 5.6 at 1 µg/ml EGF for HEK293 cells and 34% ± 6.0 at 200 ng/ml for MDA MB 231 cells (Fig. 1D).
Fig. 1. EGF treatment of HEK 293 and MDA MB 231 cells reduces the amount of TRAIL-induced apoptosis. A, HEK 293 cells (i), MDA MB 231 cells (ii), and MCF-7 cells (iii) were preincubated with or without 1 μg/ml EGF for 1 h. Where indicated, 1 μg/ml of soluble his-TRAIL was added to the culture medium. After the indicate times, the amount of apoptosis was quantitated by acridine orange staining of DNA condensation. Bars, ± SD of three separate experiments. B, MDA MB 231 cells were treated as indicated in tissue culture plates. After 24 h of treatment, the cells were visualized under a phase contrast inverted microscope equipped with a digital Spot camera. C, HEK293 cells were treated as indicated above for 48 h and stained with PI to detect DNA content. The amount of sub-G1 peak for each condition was determined by flow cytometry indicated by the gated region in each histogram. This represents the trend from three independent experiments. D, cells were treated with a range of EGF concentrations (20 ng/ml to 1 μg/ml) in the presence or absence of 1 μg/ml of TRAIL. The percentage of EGF protection was determined by percentage reduction in TRAIL-induced apoptosis. Bars, ± SD of three separate experiments.
EGF-inhibited TRAIL-induced Caspase 3-like Activation. TRAIL causes apoptosis through the activation of caspses including caspase 3 (8, 19). HEK 293 cells were treated with TRAIL in the presence or absence of EGF, and the amount of caspase 3-like activity was determined by cleavage of an Ac-DEVD-pNA peptide using a colorimetric assay described in the “Materials and Methods” section. TRAIL treatment increased the amount of caspase 3-like activity to 2.6-fold over basal levels in HEK 293 cells (Fig. 2A). Addition of caspase inhibitor Ac-DEVD-fmk to TRAIL-treated cells blocked TRAIL-induced caspase activation indicating cleavage of Ac-DEVD-pNA was attributable to caspase 3-like activity (Fig. 2A). On EGF treatment, TRAIL-induced caspase 3-like activity decreased to 1.6-fold increase over basal levels in HEK 293 cells. Similar results were also found in MDA MB 231 cells treated with TRAIL and EGF (data not shown). In addition, EGF and TRAIL treatment failed to cleave the caspase 3 substrate PARP in HEK 293 cells over a 48-h time course (Fig. 2B). However, TRAIL treatment alone did lead to the cleavage of PARP as indicated by the formation of the 85 kDa-cleaved fragment. The blots were stripped and reprobed with antibodies against MAPK phosphatase MKP-1 that is not cleaved during apoptosis (20) indicating equal amounts of protein being expressed in all of the conditions. This indicates that EGF protects against TRAIL-induced caspase 3-like activation.

EGF Blocks TRAIL-mediated Cytochrome C Release from the Mitochondria Downstream of Caspase 8 Activation and BID Cleavage. TRAIL ligation leads to the release of cytochrome c from the mitochondria (8, 9). HEK 293 and MDA MB 231 cells were treated with TRAIL alone, or EGF alone or in combination for 24 h, and the amount of cytochrome c released from the mitochondria was determined. The cells were stained with antibodies against cytochrome c and with Hoechst. In MDA MB 231 cells, control cells showed normal DNA staining, and the cytochrome c staining was punctuate indicating cytochrome c was in the mitochondria. EGF treatment alone did not alter staining patterns, whereas TRAIL treatment induced DNA condensation that is an indicator of apoptosis and a reduction in cytochrome c staining in cells as an indicator of cytochrome c release from the mitochondria. However, EGF and TRAIL treatment lead to the presence of cytochrome c in punctuate structures and normal DNA staining (Fig. 3A). This indicates that EGF significantly reduced the amount of cytochrome c released from the mitochondria. In MDA MB 231 cells, 90% ± 3.8 of the cells released cytochrome c after TRAIL treatment (Fig. 3B); however, addition of EGF and TRAIL decreased the amount of cytochrome c released to 45% ± 10.1. In untreated control and EGF treated cells, only 3.5% ± 0.8 showed cytochrome c release. TRAIL treatment caused 43% ± 3.6 of HEK 293 cells to release cytochrome c from the mitochondria compared with only 21% ± 3.4 in cells treated with both TRAIL and EGF (Fig. 3B). In addition, Western blotting for the presence of cytochrome c in the cytosol of HEK 293 cells was performed. Similar to immunohistochemical staining, cytochrome c was detected in the cytosolic fraction after TRAIL treatment over a 48-h time course peaking at 24 h. The level of cytochrome c in the cytosol was reduced after 48 h of TRAIL treatment probably because of loss of cytochrome c to the extracellular space from increased permeability of the plasma membrane during apoptosis. After pre-treatment with EGF, cytochrome c failed to be significantly detected over a 48-h time course after TRAIL treatment in the cytosol (Fig. 3C). All of the lanes had equal amounts of protein as determined by level of expression of actin. This suggests that EGF protects both HEK 293 and MDA MB 231 cells from TRAIL-induced cytochrome c release from the mitochondria.

After TRAIL ligation of its receptors, caspase 8 is cleaved into its active form (6, 8). HEK 293 cells were treated with TRAIL in the presence or absence of EGF for 24 h. The cells were lysed and Western blotted for caspase 8. After treatments with EGF alone, TRAIL alone, or with TRAIL and EGF, Western blotting revealed that the inactive form of caspase 8 was present in control and EGF-treated cells but was cleaved into its active form in TRAIL or TRAIL and EGF-treated cells (Fig. 3D). Thus, caspase 8 is activated by TRAIL in the presence of EGF. Caspase 8 activation leads to the cleavage of specific proteins. One of these proteins is the Bcl-2 family member BID (11). On cleavage, BID translocates to the mitochondria contributing to the release of cytochrome c and caspase 3-like activation (11). In HEK 293 cells treated with TRAIL alone or both EGF and TRAIL, BID was found cleaved (Fig. 3D) compared with cells untreated (control) and EGF-treated. Western blotting for MKP-1 resulted in MKP-1 being evenly expressed in all of the conditions. Thus, the reduction in caspase 8 and BID protein levels in TRAIL-treated cells was attributable to cleavage. Taken together, these results suggest that TRAIL-induced caspase 8 and BID cleavage occurs before EGF blocks TRAIL-induced mitochondrial cytochrome c release.

AKT but not ERK Activation Is Required for the Protective Effects of EGF against Apoptosis. EGF stimulates specific signal transduction pathways that have antiapoptotic properties. Both AKT and the ERK MAPK pathways are activated by EGF and have been shown to be antiapoptotic (21, 22). After EGF stimulation, AKT kinase activity increases within the first hour and peaks after 2 h (data not shown). The activation of AKT is regulated by phosphorylated phosphatidylinositols that are phosphorylated by the catalytic activity of PI3K. The pleckstir homology domain of AKT binds these phosphatidylinosito-dependent kinases (PDK1/2; Ref. 23). PI3K activation is inhibited by wortmannin that subsequently blocks AKT activation. EGF (1 μg/ml) stimulation of HEK 293 cells in the presence of 100 nM wortmannin.

Fig. 2. EGF treatments of HEK 293 cells blocked TRAIL-induced caspase 3-like activation. A, HEK 293 cells were treated with EGF, TRAIL, or in combination for 24 h. The cells were lysed, and caspase 3-like activity was determined by cleavage of the Ac-DEVD-pNA peptide. As a negative control, the caspase 3-like inhibitor Ac-DEVD-fmk (50 μM) was added to TRAIL-treated cells, and the amount of caspase 3-like activity was determined as above. Caspase activity is represented as a fold increase over basal levels. Bars, ± SD from the three experiments. B, HEK 293 cells were treated same as above and lysed. The lysate was Western blotted for the caspase-3 substrate PARP with anti-PARP antibodies over a 48-h time course. The blot was stripped and reprobed with anti-MKP-1 antibodies. Experiments were repeated three times.
blocked AKT phosphorylation that is an indicator of its active state (data not shown). HEK 293 cells were tested to determine whether inactivation of PI3K by wortmannin alters EGF protection against TRAIL-induced apoptosis. Cells were treated with 1 µg/ml EGF in the presence or absence of 100 nM of wortmannin. TRAIL (1 µg/ml) was added to these cells, and the amount of apoptosis was determined by acridine orange staining. Cells treated with wortmannin or EGF alone were 6.2% ± 3.7 and 7.7% ± 2.4, apoptotic, respectively. This is similar to the 10% ± 2.3 apoptosis found in control cells (Fig. 4A). TRAIL-treated cells had 43% ± 7.1 apoptosis, whereas EGF- and

![Fig. 3. TRAIL-mediated cytochrome c release from the mitochondria was inhibited by EGF treatment.](image)

A. MDA MB 231 cells were grown on coverslips and treated as indicated for 24 h. The cells were then fixed in formaldehyde and immunohistochemically stained for cytochrome c (red) and DNA stained with Hoechst dye (blue). The cells were visualized on a Zeiss Axiphot and images captured by a digital camera. B. HEK 293 (i) and MDA MB 231 (ii) cells were grown on coverslips and treated as described above. At least 200 cells were scored for the release of cytochrome c from the mitochondria. This was determined by the percentage of diffuse and faintly stained cytochrome c in apoptotic cells as compared with the punctuate staining of cytochrome c in normal cells. Bars, ± SD of three independent experiments. C, cytochrome c was also Western blotted for its presence in the cytosolic fraction of cells that indicates cytochrome c was released from the mitochondria. HEK293 cells were treated with TRAIL (1 µg/ml) for a 48-h time course in the presence and absence of EGF (1 µg/ml). The cells were dounce homogenized, and membrane and cytosolic fractions isolated as described in "Materials and Methods." The fractions were Western blotted for cytochrome c along with actin as a control. M, membrane fraction; S100, cytosolic fractions. D, the cells were lysed and Western blotted for the inactive full-length form of caspase 8 and for full-length BID where indicated using antiserum against these proteins. These blots were stripped and reprobed with antibodies against MKP-1 protein (fails to be cleaved during apoptosis) as a negative control. These experiments were done three times with similar results.
TRAIL-treated cells showed reduced apoptosis of 27% ± 2.8. The addition of wortmannin restored the TRAIL-induced apoptosis to 50% ± 5.8 (Fig. 4A). This suggests that PI3K activation is required for EGF survival response against TRAIL-induced apoptosis.

Activation of ERK in response to EGF is effectively inhibited by the Parke-Davis compound PD098059 (data not shown). This compound blocks the activation of the MAPK kinases MEK-1 and -2 in the RAS/ERK pathway (14). In contrast to wortmannin treatment, the addition of PD098059 did not alter the ability of EGF to inhibit TRAIL-induced apoptosis (Fig. 4B). Untreated control, EGF, and PD098059-treated cells showed similar levels of apoptosis (8% ± 2, 10% ± 1.4, and 9% ± 1, respectively), whereas TRAIL-treated cells showed 61% ± 8 apoptosis (Fig. 4B). EGF in the presence of absence of the PD098059 compound inhibited TRAIL-induced apoptosis (36% ± 4.6 and 35% ± 2.5, respectively). This suggests that EGF activation of the PI3K signaling pathway but not the RAS/ERK signaling pathway is required for the prevention of TRAIL-induced apoptosis.

Expression of myr-AKT Blocks TRAIL-induced Apoptosis. Because wortmannin reverses the effect of EGF on TRAIL-induced apoptosis, the inhibition of PI3K activation blocks EGF-mediated AKT phosphorylation, and AKT has antiapoptotic properties, the ability of AKT to block TRAIL-induced apoptosis was determined. Both HEK 293 and MDA MB 231 cells were stably transfected with vector alone, AKT KM, and myr-AKT as determined by Western blotting (Fig. 5A). These cells were treated with TRAIL for 24 h, and the amount of apoptosis determined by acridine-orange staining. TRAIL-induced apoptosis in 12.4% ± 2.3 of HEK 293 cells expressing myr-AKT compared with 26% ± 4.3 of vector alone and AKT KM cells (Fig. 5A). TRAIL-induced apoptosis in MDA MB 231 cells expressing myr-AKT was 25% ± 9.5, whereas vector alone and AKT KM-expressing cells were 49% ± 5 (Fig. 5B). The differences in the amount of TRAIL-induced apoptosis in stably transfected cell lines compared with parental cell lines are attributable to an altered susceptibility of the transfected cells to undergo TRAIL-induced apoptosis. These findings indicate that AKT activation alone is sufficient to inhibit TRAIL-induced apoptosis.

AKT KM Blocks EGF Protection of HEK 293 Cells against TRAIL-induced Apoptosis and Mitochondrial Cytochrome c Release. Expression of AKT KM has been demonstrated to act as a dominant negative blocking AKT phosphorylation of its antiapoptotic substrates in several different cell types (23–25). To determine whether expression of AKT KM blocks the ability of EGF to protect cells against TRAIL-induced apoptosis, HEK 293 cells expressing vector alone or AKT KM were treated with EGF, TRAIL, or both, and the amount of apoptosis was determined. Untreated and EGF-treated AKT KM cells showed 7.0% ± 0.6 and 7.7% ± 0.7 apoptosis and were similar to vector alone cells at 5.4% ± 2.1 and 7.7% ± 1.7 apoptosis, respectively (Fig. 6A). TRAIL induced apoptosis in 45% ± 3.4 of vector alone cells and 35% ± 0.9 in AKT KM cells. In the presence of EGF, TRAIL-induced apoptosis was reduced to 17% ± 5.4 in vector alone cells but was 40% ± 2.6 in AKT K-M cells (Fig. 6A). Constitutive active AKT-expressing cells treated with TRAIL failed to induce apoptosis in the presence or absence of EGF (data not shown). In addition, EGF treatment failed to protect cytochrome c release from the mitochondria in HEK 293 cells expressing AKT KM. Vector alone cells pretreated with EGF showed 20% protection against TRAIL-mediated mitochondrial cytochrome c release, whereas cells expressing AKT KM showed only 5% protection (Fig. 6B). This indicates that AKT KM is acting as a dominant-

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Fig. 4. Wortmannin but not PD098059 blocks EGF inhibition of TRAIL-induced apoptosis. A, HEK 293 cells were incubated with or without 1 μg/ml EGF, 1 μg/ml TRAIL, and 100 μM of wortmannin as indicated. The apoptotic index of each combination of treatments was determined. Bars, ± SD of three independent experiments. *, P < 0.05 using Student’s t test for the difference between TRAIL and TRAIL + EGF treatments. B, HEK 293 cells were incubated with or without 1 μg/ml EGF, 1 μg/ml TRAIL, and 50 μM of PD098059 as indicated. The percentage of apoptosis was determined over basal levels using acridine orange staining as described in “Materials and Methods.” +, P < 0.05 using Student’s t test for the difference between TRAIL versus TRAIL + EGF and TRAIL + EGF + PD098059 treatments, respectively. The experiments were repeated three separate times; bars, ± SD.

Fig. 5. AKT effectively blocks TRAIL-induced apoptosis. A, HEK 293 cells and MDA MB 231 cells were stably transfected with vector alone, kinase inactive AKT (AKT KM), and activated AKT (myr-AKT). B, HEK 293 and (C) MDA MB 231 cells expressing vector alone, AKT KM, or myr-AKT were treated with 1 μg/ml TRAIL for 24 h and the amount of apoptosis determined. The level of expression of AKT was determined compared with vector alone expressing HEK 293 cells. The percentage of apoptotic cells was determined over basal levels of untreated control cells. Three separate and independent experiments were performed; bars, ± SD.

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from mitochondria were determined. TRAIL where indicated for 24 h, and the amount of apoptosis and cytochrome c release was measured. The amount of cytochrome c compared with the amount of cytochrome c released in the presence of 1 μg/ml TRAIL and 1 μg/ml EGF was used to calculate the percentage of apoptosis. The percentage of apoptosis was determined over the basal level of apoptosis in control cells, bars, ± SD from three separate experiments.

B, cells were fixed and stained with antibodies against cytochrome c as described in “Materials and Methods.” The amount of cytochrome c release from the mitochondria was determined under each condition. The percentage of protection was determined as the amount of cytochrome c released in the presence of 1 μg/ml TRAIL and 1 μg/ml EGF compared with the amount of cytochrome c released after only TRAIL treatment; bars, ± SD from three separate experiments.

AKT KM Delays EGF-mediated BAD Phosphorylation. The proapoptotic Bcl2 family member BAD is phosphorylated and inactivated by AKT. On EGF stimulation, BAD becomes phosphorylated on serine 136 by AKT (26, 27). HEK293 cells transiently transfected with BAD were treated with EGF over a time course and Western blotted with an antibody specific for the phosphoserine 136 residue in BAD. The amount of BAD phosphorylation was increased over 10 min of EGF treatment peaking at 30 min (Fig. 7A). In cells expressing vector alone or AKT KM, BAD phosphorylation showed a 6-fold increase (Fig. 7B). However, AKT KM-expressing cells had a delayed response to EGF-mediated BAD phosphorylation (1 h for AKT KM versus 30 min for vector alone). BAD protein expression was found to be equal in the transfected cells as indicated by Western blotting for BAD (data not shown). This suggests that part of the protection against cytochrome c release from the mitochondria is mediated by delayed phosphorylation of BAD.

DISCUSSION

EGF stimulation of HEK 293 and the breast cancer cell lines MDA MB 231 and MCF-7 effectively protects these cells from TRAIL-induced apoptosis. EGF was shown previously to also protect cells against DR ligands TNF-α and FAS-induced apoptosis (14, 28). This suggests that EGF generally protects against DR-induced apoptosis. We have additionally delineated the mechanism of this protection against apoptosis. EGF blocks TRAIL-induced apoptosis by inhibiting mitochondrial cytochrome c release downstream of caspase 8 activation and cleavage of BID. Furthermore, EGF activation of AKT is both required and sufficient for protection against TRAIL-induced apoptosis in HEK 293 and MDA MB 231 cells possibly through BAD phosphorylation. These results provide a model of how EGF protects against TRAIL-induced apoptosis and in general against DR-induced apoptosis. EGF binds to its receptor leading to the activation of AKT and phosphorylation of AKT substrates including the Bcl2 family member BAD. This blocks TRAIL-induced apoptosis by inhibiting the release of cytochrome c from the mitochondria and inhibition of caspase 3-like activation effectively blocking TRAIL-induced apoptosis.

EGF protection against apoptosis was demonstrated in other cell types. In rat prostatic epithelial cells and cultured fetal hepatocytes, EGF protected cells from TNF-β-induced apoptosis (22, 29). The protective effect in other cells is less apparent. In fibroblasts, EGF is not able to significantly protect against UV-B induced apoptosis (30). Similarly EGF was unable to protect adipocytes from TNF-α-induced apoptosis (28). These differences could be attributable to the ability of these cells to activate AKT via EGF stimulation or the amount or type of apoptotic stimulus used. A more likely reason is different levels of expression of the various EGF receptors or the absence of EGF receptor family members in some cell lines. However, it is still unclear why EGF varies in its ability to protect against cell death.

EGF binds to ErbB1 and recruits different combinations of EGF receptor family members (2, 31). Expression of EGF receptor family members is especially relevant in breast cancer. Breast cancer cells express multiple members of the EGF receptor family including ErbB1 and ErbB2/neu (1, 4). High expression of ErbB2/neu in specific subsets of breast tumors correlates with poor prognosis. In breast cancer cell line MDA MB 453, the expression of ErbB2/neu protects cells against Taxol-induced apoptosis (13, 15). Furthermore, overexpression of ErbB2/neu protects cells against TRAIL-induced apoptosis mediated by AKT activation (32). Murine epithelial cells deficient in expression of ErbB1 are resistant to transformation after transfection of oncogenic son of sevenless or RAS mutants (33). EGF is also expressed in breast cancer tumors providing tumor cell activation of EGF receptors via autocrine and paracrine mechanisms (1). AKT is found activated in many breast cancer cell lines (34, 35). This would predict that breast cancer cells have survival signals that could be...
relevant in their sensitivity to induce apoptosis during treatment. Besides breast cancer cells, AKT also protects prostate cancer cells from TRAIL-induced apoptosis (36, 37). Our results indicated that EGF stimulation of the breast cancer cell line MDA MB 231 protects them from TRAIL-induced apoptosis by a mechanism involving EGF receptor-mediated AKT activation and blockage of mitochondrial cytochrome c release. The EGF receptors providing this protection from TRAIL-induced apoptosis are currently unknown.

EGF treatment of epithelial cells significantly reduced but did not eliminate TRAIL-induced apoptosis. Indeed, the amount of TRAIL-induced apoptosis increases over time in the presence of EGF; however, it is always less than TRAIL alone even after 72 h of TRAIL treatment (data not shown). This could be attributable to the ability of DRs including DR4 and DR5 to activate caspases independent of cytochrome c release from the mitochondria (8). In addition, the level of surface expression of ErbB receptors on the cells could contribute to this partial effect. EGF receptors and AKT are expressed at modest levels in both HEK 293 and MDA MB 231 cells (data not shown). This survival response by EGF could more closely represent the environment of tumors in which moderate changes in cells favoring survival are sufficient to allow cells to become resistant to chemotherapy.

EGF blocks TRAIL-induced apoptosis through activation of AKT. We have evidence that EGF blockage of apoptosis is through the inhibition of cytochrome c release from the mitochondria. AKT has been demonstrated to block cytochrome c release from the mitochondria through phosphorylation of substrates (23, 26, 27). Indeed, we have shown that AKT KM reverses EGF-mediated blockage of mitochondrial cytochrome c release. One of these substrates for AKT is a member of the BCL2 family, BAD, that has been shown to regulate cytochrome c release from the mitochondria. Unphosphorylated BAD is found in a complex with BCL2. On AKT-mediated phosphorylation, BAD releases BCL2 and binds to 14–3-3 protein. BCL2 then binds with BAX inhibiting the release of cytochrome c from the mitochondria (23, 26, 27). EGF treatment leads to AKT-mediated phosphorylation of BAD and expression of AKT KM that blocks EGF protection and delays the ability of AKT to phosphorylate BAD after EGF stimulation. Thus, AKT phosphorylation of BAD might be involved in the blockage of cytochrome c release from the mitochondria, thereby linking EGF signaling to TRAIL-mediated cytochrome c release. However, this involvement is only partial, because AKT KM does not block BAD phosphorylation but only delays its phosphorylation. AKT also phosphorylates other substrates that block apoptosis. Caspase 9 is phosphorylated by AKT inhibiting its apoptotic function, but caspase 9 activation occurs downstream of cytochrome c release (23) and is not likely involved in EGF survival responses. AKT could also activate transcription factors such as NFκB and cAMP-responsive element binding protein that lead to up-regulation of antiapoptotic proteins including the BCL2 family member BclxL (23, 25, 38). It is controversial if AKT activates NFκB after growth factor stimulation. It has been reported that EGF receptors activate NFκB transcriptional activity independent of AKT (39). It remains to be elucidated whether AKT activation of NFκB is important in AKT survival responses. Nevertheless, up-regulation of antiapoptotic genes such as BclxL had been shown to block cytochrome c release from the mitochondria after apoptotic stimulus. Thus, the AKT substrate(s) contributing to EGF blockage of cytochrome c release from the mitochondria remains unclear and will need to be additionally investigated.

TRAIL is proposed as a treatment for cancer, because it induces apoptosis effectively in cancer cells or in combination with chemotherapeutic agents without affecting healthy surrounding tissue (5, 6, 23, 40). Herein, we have determined that TRAIL-induced apoptosis is blocked by EGF-mediated AKT activation. Because EGF and its receptors are involved in cancer progression (1, 2), these cancers might be more resistant to TRAIL-based cancer treatments. Thus, it is important to determine the EGF responsiveness of tumors to evaluate the effectiveness of TRAIL therapy. Furthermore, this knowledge of how cells protect themselves from apoptotic stimuli will provide new targets for pharmaceuticals that could overcome the resistance to TRAIL-induced apoptosis in cancer cells. Indeed, one potential target is already in clinical use. The inhibitor of EGF receptor signaling, Hereceptin, has been shown to be effective in the treatment of breast cancer (16), and in combination with TRAIL increases the sensitivity of cells to TRAIL-induced apoptosis (41). Thus, inhibiting EGF ant apoptotic signaling in combination with TRAIL treatment might be an effective treatment for EGF-responsive cancers. This will be the focus of future studies in both cancer cells lines and mouse tumor models.

ACKNOWLEDGMENTS

We thank Drs. Arnold Greenberg, Gordon Mills, and Gary Johnson for generous gifts of reagents and evaluation of manuscript. We also thank Christine Vande Velde and Jeannick Cizeau for technical assistance.

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