Simultaneous Inhibition of Epidermal Growth Factor Receptor (EGFR) Signaling and Enhanced Activation of Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) Receptor-mediated Apoptosis Induction by an scFv:sTRAIL Fusion Protein with Specificity for Human EGFR*

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Edwin Bremer, Douwe F. Samplonius, Linda van Genne, Marike H. Dijkstra, Bart Jan Kroesen, Lou F. M. de Leij, and Wijnand Helfrich‡

From the Groningen University Institute for Drug Exploration, University Medical Center Groningen Department of Pathology and Laboratory Medicine, Section Medial Biology, Laboratory for Tumor Immunology, 9713 GZ Groningen, The Netherlands

Epidermal growth factor receptor (EGFR) signaling inhibition by monoclonal antibodies and EGFR-specific tyrosine kinase inhibitors has shown clinical efficacy in cancer by restoring susceptibility of tumor cells to therapeutic apoptosis induction. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising anti-cancer agent with tumor-selective apoptotic activity. Here we present a novel approach that combines EGFR-signaling inhibition with target cell-restricted apoptosis induction using a TRAIL fusion protein with engineered specificity for EGFR. This fusion protein, scFv425:sTRAIL, comprises the EGFR-blocking antibody fragment scFv425 genetically fused to soluble TRAIL (sTRAIL). Treatment with scFv425:sTRAIL resulted in the specific accretion to the cell surface of EGFR-positive cells only. EGFR-specific binding rapidly induced a dephosphorylation of EGFR and downstream mitogenic signaling, which was accompanied by cFLIP\textsubscript{L} down-regulation and Bad dephosphorylation. EGFR-specific binding converted soluble scFv425:sTRAIL into a membrane-bound form of TRAIL that cross-linked agonistic TRAIL receptors in a paracrine manner, resulting in potent apoptosis induction in a series of EGFR-positive tumor cell lines. Co-treatment of EGFR-positive tumor cells with the EGFR-tyrosine kinase inhibitor Iressa resulted in a potent synergistic pro-apoptotic effect, caused by the specific down-regulation of cFLIP\textsubscript{L}. Furthermore, in mixed culture experiments binding of scFv425:sTRAIL to EGFR-positive target cells conveyed a potent apoptotic effect toward EGFR-negative bystander tumor cells. The favorable characteristics of scFv425:sTRAIL, alone and in combination with Iressa, as well as its potent anti-tumor bystander activity indicate its potential value for treatment of EGFR-expressing cancers.

The epidermal growth factor receptor (EGFR)\textsuperscript{1} is a transmembrane receptor tyrosine kinase comprising an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain (1, 2). Binding of epidermal growth factor or transforming growth factor-\(\alpha\) results in EGFR dimerization and subsequent activation of the intrinsic tyrosine kinase activity. Phosphorylated EGFR concomitantly triggers downstream mitogenic signaling via both the p44/42 MAPK and PI3K pathways (3, 4). Normal EGFR signaling plays a pivotal role in organ development and repair and in the regulation of cell survival. Aberrant EGFR signaling strongly contributes to the malignant features in cancer including an increased resistance to apoptosis. Aberrant signaling can be the result of EGFR overexpression by EGFR gene amplification, which can lead to very high cell surface expression of up to 10\textsuperscript{6} EGFR molecules per tumor cell. Alternatively, various oncogenic mutations of EGFR have been described including EGFRvIII, an EGFR mutant that possesses ligand-independent tyrosine kinase activity (5) and that appears to be selectively expressed in tumor cells since it is not found in normal cells. Recently, also mutations in the EGFR-tyrosine kinase domain have been identified in a subset of lung cancer patients that appear to activate anti-apoptotic pathways (6).

Several targeted strategies have been developed to specifically inhibit aberrant EGFR signaling. Monoclonal antibodies, e.g. mAb C225 (Cetuximab) and mAb 425 (7, 8), competitively inhibit the binding of natural ligands to the extracellular ligand binding domain. Small molecule tyrosine kinase inhibitors, e.g. Iressa (also known as ZD1839 or Gefitinib) (9, 10), competitively inhibit with ATP for binding to the EGFR-tyrosine kinase domain. The clinical efficacy of these agents appears to rely on multiple anti-cancer mechanisms, including inhibition of cell cycle progression, inhibition of metastasis, and an increase in the susceptibility of cells to apoptosis. However, despite promising anti-tumor activity in clinical trials (11–14), both classes of EGFR-signaling antagonists do not appear to be curative. Therefore, additional EGFR-targeted strategies or combination with other therapeutic approaches

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‡ To whom correspondence should be addressed: University Hospital Groningen, Dept. of Pathology and Laboratory Medicine, Section Medical Biology, Laboratory for Tumor Immunology, Bldg. CMC VII, Rm. T2.228, Hanzeplein 1, 9713 GZ Groningen, The Netherlands. Tel.: 31-50-361-3733; Fax: 31-50-361-9911 E-mail: w.helfrich@med.rug.nl.

1 The abbreviations used are: EGFR, epidermal growth factor receptor; MAPK, mitogen activated protein kinase; PI3K, phosphatidylinositol 3-kinase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; memTRAIL, membrane TRAIL; sTRAIL, soluble TRAIL; scFv, single chain fragment of variable regions; cFLIP\textsubscript{L}, cellular flice inhibitory protein long; Z, benzoyloxycarbonyl; FMK, fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase.
are under investigation. In this respect strong synergistic tu-
morexic effects have been reported for strategies in which
EGFR-signaling antagonists are combined with radiation or
chemotherapy (12, 14, 15) and, more recently, with the cytokine
TRAIL (16).

TRAIL is normally present as a trimeric type II transmem-
brane protein (memTRAIL) on various immune effector cells. TRAIL spe-
cifically induces apoptosis in cancer cells (17) and virus-infected cells (18) without apparent apoptotic activity
toward normal human cells. Homotrimeric memTRAIL ini-
tiates apoptosis after cross-linking of the agonistic receptors
TRAIL-R1 and TRAIL-R2 (19–22), leading to activation of the
extrinsic apoptotic pathway via the death-inducing signaling
complex (23–30). Assembly of the death-inducing signaling
complex sequentially activates initiator caspases (caspase 8 or
10) and effector caspases (e.g., caspase 3 and 7) and ultimately
ends in apoptotic cell death. memTRAIL can be proteolytically
cleaved into a soluble form (sTRAIL). Several recombinant
forms of sTRAIL have been generated that show strong tumori-
cidal activity in vitro and in xenografted mouse tumor models
without toxic side effects (31–33). Pharmacokinetic studies in
cynomolgus monkeys and chimpanzees revealed no TRAIL-
related toxicity (34), also indicating a potential role for sTRAIL in
human cancer therapy. Nevertheless, several recent reports de-
scribed apoptotic activity of sTRAIL toward various normal hu-
man cells, including primary human hepatocytes (35), keratino-
cyes (36), prostate epithelial cells (37), and brain tissue (38).

Previously we and others showed that sTRAIL can be geneti-
cally fused to a tumor-selective antibody fragment (39, 40),
resulting in fusion proteins with enhanced and tumor-re-
stricted apoptotic activity. Here we present and analyze the
mode of action of a novel and promising strategy that combines
EGFR-signaling inhibition with target cell-restricted apoptosis
induction using a TRAIL fusion protein with engineered spec-
icity for EGFR.

EXPERIMENTAL PROCEDURES

Cell Lines

The following cell lines were purchased from the ATCC (Manassas,
VA): Jurkat (ALL T-cell line), A431 (epidermoid vulva carcinoma),
A172, HS683 (glioblastoma), SW948, and WiDr (colon carcinoma).
Cell line Jurkat.EGFRvIII was generated by electroporation of Jurkat cells
with plasmid pH6A4p-1-neo/EGFRvIII (a kind gift of Dr. D. Bigner,
Duke University Medical Center, NC), after which transfectants were
selected by G418 selection (500 µg/ml, Invitrogen). Cell lines were
cultured at 37 °C in a humidified 5% CO2 atmosphere. Jurkat, SW948,
and WiDr cells were cultured in RPMI 1640 (Cambrex Bio Science,
Verviers, France) supplemented with 15% fetal calf serum. A431,
HS683, and A172 cells were cultured in Dulbecco’s modified Eagle’s
medium, 10% fetal calf serum, and 4 mM L-glutamine (Cambrex
Bio Science).

Monoclonal Antibodies and Inhibitors

TRAIL-neutralizing mAb 2E5 was purchased from Alexis (10F,
Breda, The Netherlands). mAb 425 was provided by Merck.
mAb 425 is a murine IgG2a with high binding affinity for the extracellular domain of
both EGFR and EGFRvIII. mAb 425 blocks EGFR binding to EGFR and
competes with scFv425 for binding to the same epitope. Total caspase
inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-FMK, and caspase-9
inhibitor Z-LEHD-FMK were purchased from Calbiochem. EGFR-tyro-
sine kinase inhibitor Iressa was kindly provided by AstraZeneca Inc
(Macclesfield, Cheshire, UK). PI3K inhibitor wortmannin was pur-
chased from Sigma-Aldrich. Final working concentrations of inhibitors
were diluted in serum-free medium from a stock of 10 mM in Me2SO.

Production of scFv425:sTRAIL

Fusion protein scFv425:sTRAIL was constructed and produced es-
sentially as described previously (39). Briefly, in the first multiple
cloning site of vector pEE14, the high affinity antibody fragment
scFv425 (Vh-(G4S)3-VI format) (41), kindly provided by Merck, was
directionally inserted using the unique SfiI and NotI restriction enzyme
sites. In the second MCS a PCR-truncated 593-bp DNA fragment en-
coding the extracellular domain of human TRAIL (sTRAIL) was cloned
in-frame using restriction enzymes XhoI and HindIII, yielding plasmid
pEE14-scFv425-sTRAIL. Expression plasmid pEE14-scFv425-sTRAIL
was transfected into Chinese hamster ovary K1 cells using FuGENE 6
reagent (Roche Diagnostics) according to the manufacturer’s instruc-
tions, after which transfectants were selected by the glutamine synthe-
sis system as described (42). Single cell sorting using the MoFlo high
speed cell sorter (Cytomation, Fort Collins, CO) established clone
100F1, stably secreting 2.4 µg/ml scFv425:sTRAIL into the culture
medium.

EGFR-specific Binding of scFv425:sTRAIL

EGFR-specific binding of scFv425:sTRAIL was assessed by flow cy-
tometry using the EGFR-positive tumor cell line A431 and the EGFR-
negative cell line Jurkat. In short, 1 × 106 cells were incubated with
scFv425:sTRAIL (300 ng/ml) in the presence or absence of mAb 425 (3
µg/ml). Specific binding of scFv425:sTRAIL was detected using phos-
phatidylethanolamine-conjugated anti-TRAIL mAb B-532 (Diaclone
SAS, Besançon, France) and subsequent fluorescence-activated cell
sorting analysis using an EPICS ELITE flow cytometer (Beckman
Coulter, Mijdrecht, The Netherlands). Incubations were carried out for
45 min at 0 °C and were followed by 2 washes with serum-free medium.

Target Cell-restricted Induction of Apoptosis by scFv425:sTRAIL

Target cell-restricted induction of apoptosis by scFv425:sTRAIL was
assessed by analysis of tumor cell viability, loss of mitochondrial
membrane potential (Δφ), caspase 8 and 3 activation, and PARP cleavage/
DNA fragmentation factor degradation, as described in more detail
under “Immunoblot Analysis.” Where indicated, treatment with
scFv425:sTRAIL was performed in the presence of mAb 425 (3 µg/ml)
or mAb 2E5 (1 µg/ml).

Apoptosis Assessed by Viability Assay

Cells were precultured in a 96-well plate at a density of 3 × 104
cells/well. Subsequently, cells were treated for 16 h with the various
experimental conditions in a final volume of 200 µl. Cell viability of
adherent cell lines was determined by crystal violet staining (Sigma) as
described previously (40). Cell viability of suspension cell lines was
determined using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-
methoxy-4-sulphonphenyl)-2H-tetrazolium assay according to the
manufacturer’s recommendations (Promega Benelux b.v., Leiden, The
Netherlands). Experimental apoptosis induction was quantified as the
percentage of apoptosis induction compared with base-line apoptosis
in medium control, which was set at 0% apoptosis. Each experimental
condition consisted of six independent wells.

Apoptosis Assessed by Loss of Mitochondrial
Membrane Potential (Δφ)

Δφ was analyzed using the stain DiOC6 (Eugene, The Netherlands)
as previously described (39). Briefly, cells were precultured in a 24-well
plate at a concentration of 0.5 × 106 cells/well. Subsequently, cells were
treated for 16 h with the various experimental conditions, after which
cells were harvested and incubated for 20 min with DiOC6 (0.1 µM) at
37 °C, harvested (1000 × g, 5 min), resuspended in phosphate-buffered
saline, and assessed for DiOC6 staining using flow cytometry.

Immunoblot Analysis

Cells were precultured at 1.5 × 106 cells/well in a 6-well plate, after
which cells were incubated with scFv425:sTRAIL in the presence or
absence of mAb 425 or mAb 2E5 for the indicated time points. Cell
lysates were prepared as described previously (39). Subsequently, 30 µg of
lysat was separated by SDS-PAGE under reducing conditions and
transferred to nitrocellulose by electroblooting.

Apoptosis Signaling—Caspase activation was detected using anti-
bodies directed against caspase-8 and active caspase-3 (Cell Signaling,
Beverly, MA). PARP cleavage and DNA fragmentation factor degrada-
tion was assessed using anti-PARP mAb F2 and polyclonal anti-DNA
fragmentation factor (Santa Cruz Biotechnology Inc., Santa Cruz, CA).
Expression of c-FLIP, and Bad phosphorylation was determined using
anti-c-FLIP, mAb clone NF6 (Alexis) and polyclonal anti-phospho Bad
Ser136 antibody (Cell Signaling).

EGFR Signaling—Expression levels of total and active EGFR were
assessed using anti-total EGFR (Cell Signaling) and anti-activated
EGFR (Tyr-1173) (Santa Cruz). The MAPK signal transduction path-
way was analyzed using polyclonal anti-phospho p41/42 MAPK, and
the PI3K signal transduction pathway was analyzed using polyclonal total

EGFR-restricted Apoptosis Induction by scFv425:sTRAIL

EGFR-restricted binding of scFv425:sTRAIL was assessed by flow cy-
tometry using the EGFR-positive tumor cell line A431 and the EGFR-
negative cell line Jurkat. In short, 1 × 106 cells were incubated with
scFv425:sTRAIL (300 ng/ml) in the presence or absence of mAb 425 (3
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45 min at 0 °C and were followed by 2 washes with serum-free medium.

EGFR Signaling—Expression levels of total and active EGFR were
assessed using anti-total EGFR (Cell Signaling) and anti-activated
EGFR (Tyr-1173) (Santa Cruz). The MAPK signal transduction path-
way was analyzed using polyclonal anti-phospho p41/42 MAPK, and
and anti-phospho-AKT Thr-308 and Ser-437 (Cell Signaling). Equal protein loading was assessed using anti-actin mAb (Roche Applied Science). Specific binding was visualized using appropriate secondary Horseradish Peroxidase-conjugated antibody (DAKO Cytomation, Glostrup, Denmark) and chemiluminescence (Roche Applied Science).

Differential Quantification of Apoptosis in Target and Bystander Cells during Mixed Culture Experiments

Differential cell membrane labeling of target and bystander cells was achieved using the Vybrant multicolor cell-labeling Kit (Molecular
Synergistic Induction of Apoptosis by scFv425:sTRAIL and Iressa

Jurkat.EGFRvIII cells and A431 cells were simultaneously treated with suboptimal concentrations of scFv425:sTRAIL and Iressa. Where indicated cells were co-incubated with mAb 425 (3 μg/ml), mAb 2E5 (1 μg/ml), Z-VAD-FMK (1 μg/ml), Z-IETD-FMK (1 μg/ml), Z-LEHD-FMK (1 μg/ml), or PI3K inhibitor wortmannin (10 μM). After 16 h of treatment apoptosis was assessed by Δψ as described above. The effect of single-agent and co-treatment of scFv425:sTRAIL and Iressa on apoptotic signaling and EGFR signal transduction by PI3K and MAPK was assessed by immunoblot as described above.

RESULTS

EGFR-specific Binding of scFv425:sTRAIL—To assess whether scFv425:sTRAIL displayed specific and enhanced binding to EGFR-positive cells, A431 cells were incubated with scFv425:sTRAIL and analyzed for binding by flow cytometry. Strong binding of scFv425:sTRAIL was detected on the cell surface (Fig. 1A, solid line) that could be specifically inhibited by preincubation with parental EGFR-blocking mAb 425 (Fig. 1A, dashed line). In contrast, binding of scFv425:sTRAIL to TRAIL receptors on the cell surface of EGFR-negative Jurkat cells was barely detectable (Fig. 1B). The intensity of scFv425:sTRAIL binding directly correlated to the amount of cell surface-expressed EGFR (data not shown).

EGFR-restricted Induction of Apoptosis by scFv425:sTRAIL—Treatment of EGFR-positive tumor cell lines with scFv425:sTRAIL (300 ng/ml) potently induced apoptosis (Fig. 2A: A431, 66%; HS683, 85%; WiDr, 68%; SW948, 78%; A172, 70%; Jurkat.EGFRvIII, 82%), whereas EGFR-negative Jurkat cells were fully resistant to treatment (3%). Apoptosis was specifically inhibited when cells were co-incubated with mAb 425 or TRAIL-neutralizing mAb 2E5 during treatment with scFv425:sTRAIL (Fig. 2B). Binding of scFv425:sTRAIL to cell surface-expressed EGFR results in the reciprocal activation of agonistic TRAIL receptors in a paracrine fashion, whereby apoptotic activity can also be directed toward neighboring tumor cells that are devoid of EGFR expression. To investigate the presence of such a bystander effect, Jurkat.EGFRvIII target cells were mixed with Jurkat bystander cells (ratio 1:1) and treated with scFv425:sTRAIL. After treatment, bystander and target cells were separately assessed for apoptosis, which identified a potent bystander effect of 64% apoptosis in Jurkat bystander cells (Fig. 2C). Apoptosis in Jurkat.EGFRvIII target cells reached ~50%. In both target cells and bystander cells, apoptosis was specifically inhibited by co-treatment with mAb 425 or mAb 2E5 (Fig. 2C).

Inhibition of EGFR Signaling and Subsequent Sensitization to Apoptosis by scFv425:sTRAIL Treatment—Because scFv425:sTRAIL primarily binds via its EGFR-blocking antibody fragment scFv425, the effect of scFv425:sTRAIL treatment on EGFR signaling was determined. In A431 cells, scFv425:sTRAIL induced a rapid dephosphorylation of EGFR at Tyr-1173 within 10 min, whereas total EGFR levels remained constant (Fig. 3A). The phosphorylation of EGFR observed during normal culture conditions was most likely because of a previously described transforming growth factor-α-induced autophosphorylation loop (43). Specific inactivation of EGFR signaling was accompanied by a decrease in MAPK pathway activity, which was detected by a dephosphorylation of MAPK at 1 and 3 h of treatment (Fig. 2B). In addition, the PI3K pathway was markedly inhibited after 1 and 3 h of treatment, as measured by dephosphorylation of Akt at residues Tyr-308 and Ser-473 (Fig. 2B), whereas total Akt levels remained constant.

Resistance to apoptosis by EGFR signaling is partly mediated by its effect on the anti-apoptotic protein cFLIPL and the phosphorylation of Bad via PI3K signaling. Because PI3K signaling was inactivated by scFv425:sTRAIL treatment, cFLIPL expression and Bad phosphorylation were investigated in more detail. At the early time points of 1 and 3 h of treatment a...
decrease was detected in the expression of the anti-apoptotic caspase 8 homologue cFLIP_L (Fig. 3C) that coincided with the activation of caspase 8 (Fig. 3C). Additionally, a marked decrease was observed in phosphorylation of Bad (Fig. 3C), sensitizing the mitochondria to apoptosis.

Treatment in the presence of an excess of mAb 425 significantly inhibited EGFR signaling (Fig. 3A and B), probably due to the well established EGFR inhibitory effect of this antibody (7, 44). As expected, treatment in the presence of mAb 2E5, an antibody that specifically prevents binding of TRAIL to TRAIL-R, did not significantly affect EGFR phosphorylation levels (Fig. 3A and B). Co-incubation with mAb 425 or mAb 2E5 did not restore cFLIP_L or Bad phosphorylation to levels of untreated control (Fig. 3C).

Synergistic Induction of Apoptosis by scFv425:sTRAIL and Iressa—Previously, EGFR-signaling inhibition was shown to synergistically enhance TRAIL sensitivity (16). Therefore, the potential synergistic effects of scFv425:sTRAIL with the EGFR tyrosine kinase inhibitor Iressa were assessed on A431 cells and Jurkat. EGFRvIII-transfected Jurkat cells. Treatment of A431 cells with increasing concentrations of Iressa (250–2000 nM) and a fixed concentration of scFv425:sTRAIL (100 ng/ml) resulted in a dose-dependent synergistic increase in apoptosis (Fig. 4A). Similar results, but with lower concentrations of Iressa (50–250 nM) and scFv425:sTRAIL (80 ng/ml), were obtained for Jurkat. EGFRvIII cells (Fig. 4B). Dose-response curves of treatment with a fixed concentration of Iressa (250 and 2000 nM, respectively) and increasing concentrations of scFv425:sTRAIL (up to 100 ng/ml) revealed a potent dose-dependent increase in apoptosis in both A431 and Jurkat. EGFRvIII cells already at 20 ng/ml scFv425:sTRAIL (Fig. 4C). The synergistic pro-apoptotic activity of scFv425:sTRAIL and Iressa was potently inhibited by co-treatment with mAb 425 (Fig. 4C). Parenteral Jurkat cells subjected to the same experimental conditions were fully resistant to treatment (Fig. 4D). In control experiments with the solvent Me₂SO alone or in combination with scFv425:sTRAIL, no significant induction of apoptosis was detected (data not shown).

Synergistic Induction of Apoptosis by scFv425:sTRAIL and Iressa Is Caspase 8-mediated—Next, the mechanism underlying the synergistic pro-apoptotic effect was investigated. Treatment of A431 cells and Jurkat. EGFRvIII cells with scFv425:sTRAIL and Iressa did not significantly alter TRAIL receptor expression (data not shown). Using specific caspase inhibitors, induction of apoptosis was found to be largely caspase 8-dependent since the specific caspase 8 inhibitor Z-IETD-FMK inhibited apoptosis to levels observed for Iressa alone (Fig. 5A). Caspase 9 inhibition by Z-LEHD-FMK only had a minimal effect. Immunoblot analysis further revealed a strong activation of both caspase 8 and 3, resulting in PARP cleavage within 3 h of treatment with scFv425:sTRAIL and Iressa (Fig. 5B). Single agent treatment only marginally activated caspase 8 and caspase 3 (Fig. 5B). Similar results were obtained when A431 cells were treated with scFv425:sTRAIL and Iressa (Fig. 5C). The appearance of apoptotic features was specifically inhibited when treatment was performed in the presence of mAb 425 (Fig. 5, B and C).

Inhibition of EGFR Signaling by Co-treatment with scFv425:sTRAIL and Iressa—Combination treatment of Jurkat. EGFRvIII cells with scFv425:sTRAIL and Iressa resulted in PI3K inactivation within 2 h in Jurkat. EGFRvIII, as measured by Akt dephosphorylation at Ser-473 (Fig. 6A). No inhibition of MAPK signaling was observed in Jurkat. EGFRvIII cells, which is in line with a previous report showing that EGFRvIII spe-
cifically regulates PI3K activity (45). At the concentrations used single agent treatment had no effect on mitogenic signaling in Jurkat.EGFRvIII cells (Fig. 6A). The role of PI3K inhibition in the observed synergistic apoptotic effect on Jurkat.EGFRvIII cells was further analyzed by treatment with scFv425:sTRAIL in the presence of PI3K inhibitor wortman- nin. This experiment resulted in levels of apoptosis comparable with those observed for treatment with scFv425:sTRAIL and Iressa (Fig. 6C). For A431 cells, no effect of single agent and co-treatment was detected on PI3K signaling (Fig. 6A). Single agent treatment with Iressa markedly inhibited MAPK signaling, whereas scFv425:sTRAIL treatment alone only had a minimal effect. Combination treatment of A431 cells also inhibited MAPK signaling, but only to an extent comparable with Iressa treatment alone (Fig. 6B).

Treatment with scFv425:sTRAIL and Iressa Induces c-FLIPL Down-regulation—Simultaneous treatment with scFv425:sTRAIL and Iressa markedly reduced the expression of c-FLIP L in both Jurkat.EGFRvIII and A431 cells (Fig. 6D). To a lesser extent treatment with Iressa alone down-regulated c-FLIP L in A431 cells, whereas in Jurkat.EGFRvIII cells no effect of single agent treatment was detected. Treatment in the presence of mAb 425 prevented down-regulation of c-FLIP L in both cell lines.

**DISCUSSION**

Here we describe a novel therapeutic approach in which EGFR-signaling inhibition is combined with target cell-restricted apoptosis induction using the new fusion protein scFv425:sTRAIL. Fusion protein scFv425:sTRAIL, comprising the EGFR-blocking antibody fragment scFv425 genetically fused to sTRAIL, clearly demonstrated accretion at the cell surface of EGFR-positive cells, which was specifically abrogated by preincubation with parental EGFR-blocking mAb 425.

Previously, we demonstrated that eukaryotically expressed scFv:sTRAIL fusion proteins are produced as a soluble homogeneous trimer (39). Trimeric scFv425:sTRAIL contains three identical antibody fragment domains and will, therefore, benefit from an associated enhanced avidity effect. Enhanced avidity has been shown to improve the in vivo tumor-targeting efficacy in several antibody-based strategies (46, 47). Indeed, relatively high concentrations of the parental mAb 425 were required to competitively inhibit specific binding of scFv425:sTRAIL.

Treatment with scFv425:sTRAIL potently induced apoptosis in EGFR-positive tumor cells that was specifically abrogated by co-incubation with parental mAb 425. Interestingly, the appearance of apoptotic features, such as processing of caspase 8,
was preceded by the specific dephosphorylation of EGFR and coincided with dephosphorylation of the PI3K signal transduction pathway and to a lesser extent the MAPK signal transduction pathway.

This rapid inactivation of EGFR signaling clearly points to a role for EGFR inhibition in scFv425:sTRAIL-induced apoptosis. One of the main regulators of TRAIL sensitivity, the anti-apoptotic caspase-8 homologue cFLIPL (48-50), has previously been shown to be regulated by PI3K signaling (51, 52). In A431 cells, inactivation of PI3K signaling was accompanied by a decrease in expression of cFLIPL after 1 and 3 h of treatment. Besides regulating cFLIPL expression, PI3K signaling also influences the phosphorylation status of Bad (53, 54). In A431 cells, a marked dephosphorylation of Bad was detected after 1 and 3 h. Therefore, inhibition of PI3K signaling appears to facilitate caspase 8 activation by down-regulating cFLIPL, and sensitizes the mitochondria to induction of apoptosis by dephosphorylation of Bad.

Next to PI3K inhibition, dephosphorylation of the MAPK signal transduction pathway was also detected after 1 and 3 h of treatment with scFv425:sTRAIL. Previously, MAPK activation was shown to protect against TRAIL-induced apoptosis by a mechanism occurring at or above the level of caspase 8 processing that did not involve cFLIPL (55). Conversely, although not formally proven here, MAPK inhibition can sensitize tumor cells toward scFv425:sTRAIL-induced apoptosis at or above the level of caspase 8 processing.

From these data a model for the apoptotic activity of
EGFR-restricted Apoptosis Induction by scFv425:sTRAIL

scFv425:sTRAIL can be formulated (for schematic representation see Fig. 7). First, binding of scFv425:sTRAIL leads to accretion at the cell surface of EGFR-positive tumor cells only. Subsequently, EGFR-specific binding inhibits EGFR mitogenic signaling via PI3K and MAPK and, thereby, sensitizes tumor cells to apoptosis by down-regulation of c-FLIPL and Bad phosphorylation. Concomitantly, membrane-bound scFv425:sTRAIL induces apoptosis by reciprocal cross-linking of agonistic TRAIL receptors on neighboring EGFR-positive tumor cells. Paracrine activation of TRAIL receptors by scFv425:sTRAIL is not necessarily restricted to EGFR-positive tumor cells but can also be directed toward neighboring tumor cells that have lost EGFR expression. In a recent report, we described a potent anti-tumor bystander effect for an scFv:sTRAIL fusion protein with specificity for the carcinoma-associated cell surface target antigen EG2 (56). Here, we show that scFv425:sTRAIL potently induced apoptosis in EGFR-negative bystander Jurkat cells in mixed culture experiments with Jurkat.EGFRVIII target cells. This potent anti-tumor bystander effect might help to eliminate EGFR-negative tumor cell mutants that can escape from conventional antibody-mediated strategies.

In a recent report the synergistic effect of the combined treatment with anti-EGFR monoclonal antibody cetuximab and the EGFR-specific tyrosine kinase inhibitor Iressa was described (57). We analyzed combination treatment of scFv425:sTRAIL and Iressa for the first time on a recombinant fusion protein that combines the tumoricidal effect of EGFR signal inhibition with target cell-restricted apoptosis induction. The unique characteristics of scFv425:sTRAIL described here indicate its potential therapeutic value alone and in combination with EGFR-tyrosine kinase inhibitor Iressa for the treatment of EGFR- and EGFRVIII-expressing human cancers.

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REFERENCES

1. Wells, A. (1999) Int. J. Biochem. Cell Biol. 31, 637–643
2. Holbro, T., Civenni, G., and Hynes, N. E. (2003) Exp. Cell Res. 284, 99–110
3. Glyyreve, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2009) EMBO J. 19, 3159–3167
4. Yarden, Y., and Sliwkowski, M. X. (2001) Nat. Rev. Mol. Cell. Biol. 2, 127–137
5. Wang, A. J., Ruppert, J. M., Bigner, S. H., Grzeschik, C. H., Humphrey, P. A., Bigner, D. S., and Vogelstein, B. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2965–2969
6. Lynch, T. J., Bell, D. W., Sordella, R., Gurubhagavatula, S., Okimoto, R. A., Brannigan, B. W., Harris, P. L., Haseltai, S. M., Supko, J. G., Halushka, F. G., Louis, D. N., Christiani, D. C., Settleman, J., and Haber, D. A. (2004) N. Engl. J. Med. 350, 2129–2139
7. Rodosek, U., Herlyn, M., and Koprowski, H. (1987) J. Cell. Biochem. 35, 515–520
8. Gabler, B., Aicher, T., Heiss, P., and Senekowitsch-Schmidtke, R. (1997) Anticancer Res. 17, 3157–3159
9. Normanno, N., Maielli, M. R., and De Luca, A. (2003) Cell. Physiol. 194, 131–149
10. Barker, A. J., Gibson, K. H., Grundy, W., Godfrey, A. A., Barlow, J. J., Healy, M. P., Woodburn, J. R., Ashton, S. E., Curry, B. J., Scarlett, L., Henthorn, L., and Richards, L. (2003) Bioorg. Med. Chem. Lett. 11, 1911–1914
11. Chan, K. C., Knox, W. F., Gee, J. M., Morris, J., Nicholson, R. I., Potten, C. S., and Bundred, N. J. (2002) Cancer Res. 62, 122–129
12. Raben, D., Hedrich, B. A., Chan, D., Johnson, G., and Bunn, P. A. Jr. (2002) Semin. Oncol. 29, 37–46
13. Herbst, R. S., and Hong, W. K. (2002) Semin. Oncol. 29, 18–30
14. Herbst, R. S., and Langer, C. J. (2002) Semin. Oncol. 29, 27–36
15. Solomon, R., Hapke-Kyriakos, J., Trybett, M. K., Stucker, S. A., McArthur, G. A., and Cullinan, C. (2003) Int. J. Radiat. Oncol. Biol. Phys. 55, 713–723
16. Park, S. Y., and Seol, D. W. (2002) Biochem. Biophys. Res. Commun. 295, 515–518
17. Wiley, S. L., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, K. H., Rutter, G. L., Blackwood, A. J., Rutter, C. M., and Goodwin, R. G. (1995) Immunity 3, 673–682
18. Segler, L. M., Shaues, D. M., Blanton, R. A., Peschon, J. J., Goodwin, R. G., Cosman, D., and Wiley, S. R. (1999) J. Immunol. 163, 920–926
19. Pan, G., O’Rourke, K., Chinnaiyan, A. M., Gentz, R., Ebner, N., Ni, J., and Dixit, V. M. (1997) Science 276, 111–113
20. Pan, G., Ni, J., Wei, Y. F., Yu, G., Gentz, R., and Dixit, V. M. (1997) Science 277, 815–818
21. Walczak, H., Degli-Esposti, M. A., Johnson, R. S., Smolak, P. J., Waugh, J. Y., Bionai, N., Timour, M. S., Gerhart, M. A., Schooley, K. A., Smith, C. A., Goodwin, R. G., and Rauch, C. T. (1997) EMBO J. 16, 5386–5397
22. Griffith, T. S., and Lynch, D. H. (1998) Curr. Opin. Immunol. 10, 559–563
23. Kischkel, F. C., Hellbard, S., Behrmann, I., Germer, M., Pavlin, J., Krammer, P. H., and Peter, M. E. (1995) EMBO J. 14, 5579–5588
24. Peter, M. E. (2000) Cell Death Differ. 7, 759–760
25. Kischkel, F. C., Lawrence, A. R., Ashkenazi, A., Schow, P., Kim, K. J., and Ashkenazi, A. (2001) Immunity 12, 611–620
26. Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Joo, P., Blenis, J., Krammer, P. H., and Walczak, H. (2000) Immunity 12, 599–609
27. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
28. Sprick, M. R., Rieser, E., Stahl, H., Grosse-Wilde, A., Weigand, M. A., and Ashkenazi, A. (2001) Science 295, 713–723
29. Wang, J., Chun, H. J., Wang, W., Spence, D. M., and Lenardo, M. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13884–13888
30. Ashkenazi, A., Pai, R. C., Feng, S., Leung, S., Lawrence, D. A., Marsters, S. A., Blackie, C., Chang, L., McMurtrey, A. E., Hebert, A., DeForge, L., Keume-
EGFR-restricted Apoptosis Induction by scFv425:sTRAIL

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nis, I. L., Lewis, D., Harris, L., Bussiere, J., Koeppen, H., Shahrrokh, Z., and Schwall, R. H. (1999) J. Clin. Investig. 104, 155–162
32. Roth, W., Isenmann, S., Naumann, U., Kugler, S., Bahr, M., Dichgans, J., Ashkenazi, A., and Weller, M. (1999) Biochem. Biophys. Res. Commun. 265, 479–483
33. Waleczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chiu, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P., Goodwin, R. G., Rauch, C. T., Schuh, J. C., and Lynch, D. H. (1999) Nat. Med. 5, 157–163
34. Kelley, S. K., Harris, L. A., Xie, D., DeForge, L., Totpal, K., Bussiere, J., and Fox, J. A. (2001) J. Pharmacol. Exp. Ther. 299, 31–38
35. Jo, M., Kim, T. H., Seol, D. W., Esplen, J. E., Dorko, K., Billiar, T. R., and Strom, S. C. (2000) Nat. Med. 6, 564–567
36. Leverkus, M., Neumann, M., Mengling, T., Rauch, C. T., Brocker, E. B., Kramer, P. H., and Walczak, H. (2000) Cancer Res. 60, 553–559
37. Nesterov, A., Ivashchenko, Y., and Kraft, A. S. (2002) Oncogene 21, 1135–1140
38. Nitsch, R., Bechmann, I., Deisz, R. A., Haas, D., Lehmann, T. N., Wendling, U., and Zipp, F. (2000) Lancet 356, 827–828
39. Muller, K. M., Arndt, K. M., and Pluckthun, A. (1998) FEBS Lett. 432, 45–49
40. Cockett, M. I., Bebbington, C. R., and Yarranton, G. T. (1990) Bio/Technology 8, 662–667
41. Van de Vijver, M. J., Kumar, R., and Mendelsohn, J. (1991) J. Biol. Chem. 266, 7503–7508
42. Bodeck, U., Herlyn, M., Herlyn, D., Molthoff, C., Atkinson, B., Varello, M., Steplewski, Z., and Koprowski, H. (1987) Cancer Res. 47, 3692–3696
43. Moscatello, D. K., Holgado-Madruga, M., Emlet, D. R., Montgomery, R. B., and Wong, A. J. (1998) J. Biol. Chem. 273, 200–206
44. Korti, A. A., Dolezal, O., Power, B. E., and Hudson, P. J. (2001) Biomol. Eng. 18, 95–108
45. Power, B. E., and Hudson, P. J. (2000) J. Immunol. Methods 242, 193–204
46. Kim, K., Fisher, M. J., Xu, S. Q., and El Deiry, W. S. (2000) Clin. Cancer Res. 6, 335–346
47. Jonsson, G., Paulie, S., and Grandien, A. (2003) Anticancer Res. 23, 1213–1218
48. Chang, D. W., Xing, Z., Pan, Y., Algeciras-Schimnich, A., Barnhart, B. C., Yash-Ohab, S., Peter, M. E., and Yang, X. (2002) EMBO J. 21, 3704–3714
49. Panka, D. J., Mano, T., Suhara, T., Walsh, K., and Mier, J. W. (2001) J. Biol. Chem. 276, 6893–6896
50. Suhara, T., Mano, T., Oliveira, B. E., and Walsh, K. (2001) Circ. Res. 89, 13–19
51. Zhao, S., Konopleva, M., Cabreira-Hansen, M., Xie, Z., Hu, W., Milella, M., Estrov, Z., Mills, G. B., and Andreeff, M. (2004) Leukemia 18, 267–275
52. Tran, S. E., Holmström, T. H., Ahonen, M., Kahari, V. M., and Eriksson, J. E. (2001) J. Biol. Chem. 276, 16484–16490
53. Bremer, E., Samplonius, D., de Leij, L., and Helfrich, W. (2004) Int. J. Cancer 109, 281–289
54. Muller, K. M., Arndt, K. M., and Pluckthun, A. (1999) FEBS Lett. 432, 45–49
55. Matar, P., Rojo, F., Cassia, R., Moreno-Bueno, G., Di Cosimo, S., Tabernero, J., Guzman, M., Rodriguez, S., Arribas, J., Palacios, J., and Baselga, J. (2004) Clin. Cancer Res. 10, 6487–6501
56. Osaki, M., Kase, S., Adachi, K., Takeda, A., Hashimoto, K., and Ito, H. (2004) J. Cancer Res. Clin. Oncol. 130, 8–14
57. Tran, S. E., Holmström, T. H., Ahonen, M., Kahari, V. M., and Eriksson, J. E. (2001) J. Biol. Chem. 276, 16484–16490
58. Bremer, E., Samplonius, D., Jan Kroesen, B., van Genne, L., de Leij, L., and Helfrich, W. (2004) Neoplasia 6, 636–645
59. Matar, P., Rojo, F., Cassia, R., Moreno-Bueno, G., Di Cosimo, S., Tabernero, J., Guzman, M., Rodriguez, S., Arribas, J., Palacios, J., and Baselga, J. (2004) Clin. Cancer Res. 10, 6487–6501