Reorganization of ErbB Family and Cell Survival Signaling after Knock-down of ErbB2 in Colon Cancer Cells*

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Yi Peter Hu‡§, Srinivas Venkateswarlu, Natalia Sergina, Gillian Howell‡, Patricia St. Clair‡, Lisa E. Humphrey‡, Wenhui Li‡, Jennie Hauser‡, Elizabeth Zborowska**, James K. V. Willson**, and Michael G. Brattain‡ ‡‡

From the §Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263, the Department of Surgery, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284, the **Department of Medicine and Case Western Reserve University/Ireland Cancer Center, Case Western Reserve University, Cleveland, Ohio 44106, and the §Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43689

The role of the ErbB family in supporting the malignant phenotype was characterized by stable transfection of a single chain antibody (ScFv5R) against ErbB2 containing a KDEL endoplasmic reticulum retention sequence into GEO human colon carcinoma cells. The antibody traps ErbB2 in the endoplasmic reticulum, thereby down-regulating cell surface ErbB2. The transfected cells showed inactivation of ErbB2 tyrosine phosphorylation and reduced heterodimerization of ErbB2 and ErbB3. This resulted in greater sensitivity to apoptosis induced by growth deprivation and delayed tumorigenicity in vivo. Furthermore, decreased heterodimerization of ErbB2 and ErbB3 led to a reorganization in ErbB function in transfected cells as heterodimerization between epidermal growth factor receptor (EGFR) and ErbB3 increased, whereas ErbB3 activation remained almost the same. Importantly, elimination of ErbB2 signaling resulted in an increase in EGFR expression and activation in transfected cells. Increased EGFR activation contributed to the sustained cell survival in transfected cells.

Protein-tyrosine kinases are involved in the regulation of cell growth and transformation. The binding of a ligand to the extracellular domain of a receptor tyrosine kinase induces receptor dimerization, activation of the intracellular kinase domain, and autophosphorylation (1). Tyrosine-phosphorylated residues serve as high affinity binding sites for Src homology 2-containing proteins and allow for the modulation of intracellular pathways (2, 3). Constitutive activation of these pathways is apparent in many malignancies and provides maintenance of the malignant phenotype as well as a viable target for cancer therapy.

A number of receptor tyrosine kinase subclasses have been described, among which the ErbB family members are of particular interest because of their frequent involvement in human cancer (4, 5). Four members of this family are currently known: the epidermal growth factor (EGF)1 receptor (EGFR/ErbB1), ErbB2 (also called Neu/HER2), ErbB3, and ErbB4 (6). All of them share a similar primary structure, but they differ in their ligand specificity and kinase functions (7, 8). Increased expression of EGFR is associated with relatively aggressive tumors of the stomach, bladder, lung, and breast (9). An abnormal level of EGFR has been correlated with tumor size and stage in head and neck cancer (10–12). In this type of cancer, transforming growth factor-α and EGFR are both up-regulated (13). In patients with colon carcinoma, increased EGFR mRNA in the tumors is associated with a higher rate of liver metastasis (14). Previous work in this laboratory indicated that inappropriate expression of EGFR and its ligand, transforming growth factor-α, associates with neoplastic transformation and induces malignant progression of human colon carcinoma (15, 16) as well as activation of ErbB2 (17).

Receptor-receptor interactions between ErbB family members were first described by Stern and Kamps (18) and by Wada et al. (19) for EGFR and ErbB2. Ligand-induced receptor dimerization of EGFR is believed to provide an allosteric regulatory signal coupled to kinase activation (20). However, ligand-induced heterodimerization of ErbB family members also occurs, and transmodulation of family members is also observed (8, 21–25). Transmodulation is regarded as providing more complex and flexible downstream signal transduction than homodimerization (26, 27). Heregulin-stimulated phosphorylation of both ErbB2 and ErbB3 occurs in cells coexpressing these proteins, and although ErbB2 itself does not bind heregulin, ErbB2 and ErbB3 heterodimerize to form a high affinity heregulin receptor complex in association with transphosphorylation (21). Moreover, ErbB3 appears to lack kinase activity and is therefore phosphorylated through ErbB2 transmodulation (8, 21, 28–32). Furthermore, cells coexpressing EGFR and ErbB3 show an EGF-dependent ErbB3 phosphorylation, which indicates the heterodimerization and transactivation of ErbB3 (33–35). EGFR heterodimers (EGF/ErbB2, EGFR/ErbB3) also play a major role in regulating downstream signaling in MCF-7 cells (36).

Gene amplification and overexpression of ErbB2 are correlated with poor prognosis in breast and ovarian cancer (37, 38),

1 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ErbB, protein-tyrosine kinases related to the epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; MAbs, monoclonal antibody; PARP, poly(ADP-ribose) polymerase; Qd, quiescent day; tTA, tetracycline-controlled transactivator; JOE, 6-carboxy-4′,5′-dichloro-2′,7′-dimethydroxyfluorescein.
but the role of ErbB2 in colon cancer is largely unknown. Our work has suggested a role for ErbB2 in colon cancer because coexpression of ErbB2 and heresulin in colon tumors and cell lines was demonstrated, indicating that constitutive activation of “normal” levels of ErbB2 could provide the signaling basis of tumor growth (39).

In this report, we have studied the role of ErbB2 in supporting colon tumor growth using GEO cells, which have been shown to have both autocrine transforming growth factor-α and autocrine heresulin loops (39). ScFv5R is a single chain antibody directed to the extracellular domain of ErbB2. This antibody has the endoplasmic reticulum (ER) retention signal, KDEL, at the C terminus. Therefore, when expressed ectopically, it traps ErbB2 in the ER and prevents the appearance of ErbB2 on the plasma membrane. This in turn leads to a functional inactivation of the receptor and reversion of ErbB2-induced transformation in vitro (40).

We report that ErbB2 activation was reduced significantly in ScFv5R-transfected GEO colon cancer cells, and tumorigenicity was inhibited. Blockade of ErbB2 in colon cancer cells also resulted in enhanced EGFR activation as well as changes in heterodimerization among ErbB family members in association with alterations in cell survival signaling. The compensatory changes in the ErbB family after inhibition of one of its members are of potential importance in optimizing current EGFR and ErbB2-directed therapies in cancer.

MATERIALS AND METHODS

Cell Culture—The GEO human colon carcinoma cell line was maintained continuously in a chemically defined serum-free medium (41, 42). The standard maintenance medium (designated “SF”) consists of McCoy’s 5A medium (Sigma) supplemented with pyruvate, vitamins, amino acids, antibiotics, 20 µg/ml insulin (Sigma), 4 µg/ml transferrin (Sigma), and 10 ng/ml EGF (R&D Systems, Minneapolis). The supplemented McCoy’s medium (designated “SM”) is McCoy’s 5A medium without any exogenous growth factors. Working cultures were maintained at 37 °C in a humidified atmosphere of 5% CO2 and checked routinely for mycoplasma contamination. Cells were subcultured using 0.125% trypsin (Invitrogen) in Jokil’s tissue culture medium (Invitrogen) containing 0.1% EDTA and replated into SF medium. To isolate proteins and DNAs for biochemical assays, cells were plated into 100-mm tissue culture dishes and grown in SF medium. At 80% confluence cells were changed from SF to SM medium to induce growth arrest and quiescence. Quiescence was previously operationally defined as stable minimal steady state [3H]thymidine incorporation (43). This required 3–5 days exposure to medium without growth factors (SM medium). Cells were harvested for different assays at various times leading up to 5 days of growth factor deprivation (43). In some cases, cells were harvested at day 6 after starvation.

Antibodies—Anti-ErbB2, anti-ErbB3, anti-ERK polyclonal antibodies, and anti-poly(ADP-ribose) polymerase (PARP) antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin was from Sigma. Anti-phosphotyrosine monoclonal antibody clone 4G10 and anti-phospho-EGFR Y1173 and anti-EGFR MAb225 were from Calbiochem. Caspase-3 polyclonal, anti-phospho-eIF2α (Ser-51) and eIF2α antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA).

ScFv5R Expression Vector Construction and Transfection—The ScFv5R (a single chain antibody against ErbB2) cDNA was subcloned into a tetracycline-controllable expression system kindly provided by Dr. H. Bujard at University of Heidelberg, Germany (44). As described previously (45), this system includes a tetracycline-controlled transac-
tritator (tTA), which is generated by fusing the tetracycline repressor with the activating domain of virion protein 16 of herpes simplex virus, and an expression vector that consists of a tetracycline operator sequence as well as a minimal promoter sequence derived from the human cytomegalovirus promoter. When the tetracycline repressor of the tTA binds to tetracycline operators, the virion protein 16 domain of the tTA can activate the minimal promoter to start transcription. Tetracycline can block this activation by preventing the tTA from binding to the tetracycline operator sequence. A neomycin-resistant gene under control of the mouse β-globin promoter was subcloned into the tTA-containing plasmid. This plasmid (designated pHUD15-1, 4 μg) and the ScFv5R expression vector (designated pHUD10-3, 16 μg) were linearized and cotransfected into GEO cells. Electroporation was carried out at 250 V, 960 microfarads, with a Gene Pulser (Bio-Rad). The transfected cells were allowed to grow for 2 days in normal medium (SF medium) before being subjected to selection with 620 μg/ml G418 sulfate (Mediatech, Inc., Herndon, VA). Stable cell clones resistant to G418 were ring cloned after 2–3 weeks and expanded further for screening of the mobility shift of ErbB2 as described previously (46).

Western Blotting Analysis—Cells were washed twice with cold phosphate-buffered saline and harvested in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 25 μg/ml aprotinin, 25 μg/ml trypsin inhibitor, 25 μg/ml leupeptin). Crude cell lysates were passed through a 21-gauge needle four times to shear DNA and lysed for 30 min at 4 °C. Cell lysates were then cleared by centrifugation at 12,000 rpm for 20 min at 4 °C and quantitated by Bio-Rad protein assay. Cell lysates were heated for 5 min at 95 °C in 2× sample buffer (100 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 0.04% bromphenol blue, and 2% β-mercaptoethanol). 100 μg of total protein was subjected to SDS-PAGE (7.5% acrylamide) and electrophoretically transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (Amer sham Biosciences). The membranes were blocked in TTBS buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween 20) containing 5% nonfat dried milk for 1 h at room temperature followed by incubation with primary antibody for 1 h at room temperature or overnight at 4 °C. The blots were labeled with peroxidase-conjugated AffiniPure goat antimouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) or anti-goat IgG (Santa Cruz) for 1 h, and the protein was detected using an ECL method according to the manufacturer’s instructions (Amersham Biosciences).

Immunoprecipitation—This experiment was carried out as described previously (49) with minor modifications. Briefly, the cell lysates were immunoprecipitated with different antibodies overnight at 4 °C. Immunocomplexes were absorbed by protein A-agarose (Invitrogen) for 1 h at 4 °C and washed twice with lysis buffer. Bound proteins were released by heating for 5 min at 95 °C in sample buffer. The immunoprecipitates were resolved by SDS-PAGE, then transferred to nitrocellulose and subjected to Western blot analysis with detection by the ECL method.

Quantitative PCR—RNA was collected from the appropriate cells using the Qiagen RNeasy Mini-Kit (Qiagen, Valencia, CA). A reverse transcription reaction was then performed on the RNA, and quantitative PCR was used to analyze the samples using the Taqman gene expression system following the manufacturer’s instructions (Applied Biosystems). The BiP primer-probe mix was purchased from Applied Biosystems, and glyceraldehyde-3-phosphate dehydrogenase-JOE probe was used as endogenous control. Data were analyzed using the SDS 2.2 software from Applied Biosystems.

DNA Fragmentation Assay (DNA Laddering and Cell Death Detection ELISA)—Cells were incubated on ice in DNA lysis buffer (10 mM Tris [pH 7.5], 20 mM EDTA, 0.5% Triton X-100) for 30 min. Proteins were digested overnight at 37 °C with protease K (0.4 mg/ml) and SDS (0.5%). DNA was then extracted with phenol-chloroform and precipitated with ethanol. The concentration and purity of DNA were determined by measuring UV absorbance at 260 and 280 nm. Ten micrograms of DNA from each sample was analyzed by 1.6% agarose gel electrophoresis.

For cell death detection ELISA, cells were plated in a density of 8,000 cell per well in SF medium in 96-well plates, they were subsequently allowed to attach overnight at 37 °C. The next day, media were changed and cells were treated with either AG1478 or MAh225 for another 48 h at 37 °C. DNA fragmentation was detected by using Roche Cell Death Detection ELISA kit according to the manufacturer’s instructions (Roche Applied Science).

Analysis of PARP—Cells from different stages were washed with phosphate-buffered saline, collected by scraping, and lysed with buffer containing protease inhibitors as described above. 100 μg of total protein was subjected to SDS-PAGE (7.5% acrylamide) and subsequently...
**RESULTS**

**ErbB2 Expression and Activation in ScFv5R-transfected GEO Cells**—We stably transfected ScFv5R (a single chain antibody against ErbB2) cDNA under tetracycline-repressible control into GEO cells. A typical positive clone (designated GEO C) with faster migration of the ErbB2 protein in SDS-PAGE was selected for study (Fig. 1A). The faster migration is the result of underglycosylation of ErbB2, which was trapped in the ER by the antibody in ScFv5R-transfected cells (40). The addition of tetracycline in the culture medium resulted in substantial reversal of the mobility shift of ErbB2 in SDS-PAGE (Fig. 1A, far left lane), indicating that the tetracycline-regulatable expression system was functional in GEO cells. However, total ErbB2 expression level did not change (Fig. 1A). The transfection was further confirmed by examining the activation of ErbB2 in GEO C and parental GEO cells. The phosphorylation of ErbB2 was reduced significantly in GEO C compared with parental GEO cells (Fig. 1B).

**ErbB2/ErbB3 Heterodimer Formation and ErbB3 Activation**—Heterodimer formation between ErbB2 and ErbB3 was determined by immunoprecipitation with ErbB2 followed by Western analysis of ErbB3. Immunoprecipitation by antibody to ErbB2 was utilized because others have suggested reduced accessibility of ErbB3 after heterodimerization (50). We found that ErbB2/ErbB3 heterodimerization decreased in GEO C, and this was reversed by addition of tetracycline in the culture medium (Fig. 2A). Although the ErbB2 transphosphorylation function on ErbB3 has been reduced because both ErbB2 activation and ErbB2/ErbB3 heterodimerization were reduced after transfection, Fig. 2B shows that ErbB3 activation was increased slightly in GEO C compared with the control cells. These results suggested that redistribution of ErbB3 among the ErbB family members occurred after ErbB2 was sequestered in the ER.

Activation through heterodimerization is thought to play an important role in signal transduction by receptor tyrosine kinases. ErbB3 may also be activated through heterodimerization.
tion with receptors other than ErbB2. Thus we investigated the relationships between the ErbB receptors with respect to heterodimerization as a function of the cells' autocrine activity during quiescence. Quiescence was induced by exposing the cells to the medium without growth factors (SM medium) for 5 days as described under "Materials and Methods." Death detection ELISAs were performed, and absorbance was measured at 405 nm (mean ± S.D.; n = 3). Quiescent GEO and GEO C cell lysates treated with different AG1478 and MAb225 concentrations were probed with anti-phospho-EGFR Y1173 antibody and PARP antibody by Western blotting. Actin was used as loading control. Data shown are representative of three independent experiments.

**Fig. 4. Increased EGFR activation contributes to GEO C cell survival.** GEO and GEO C cells were seeded at 8,000 cells/well in 96-well plates and allowed to adhere overnight. The next day, cells were treated with either AG1478 (A) or MAb225 (B) as described under "Materials and Methods." Death detection ELISAs were performed, and absorbance was measured at 405 nm (mean ± S.D.; n = 3). Quiescent GEO and GEO C cell lysates treated with different AG1478 and MAb225 concentrations were probed with anti-phospho-EGFR Y1173 antibody and PARP antibody by Western blotting. Actin was used as loading control. Data shown are representative of three independent experiments.

out any effects of exogenous growth factors. The determination of heterocomplexes involving EGFR was performed using an antibody specific for the EGFR which does not cross-react with other family members. ErbB2 or ErbB3 heterodimerization to EGFR was detected by Western blot analysis of the anti-EGFR immunoprecipitates. The formation of EGFR/ErbB2 het-
erodimer decreased in GEO C compared with GEO cells as a result of decreased ErbB2 activation (data not shown). In contrast, EGFR/ErbB3 heterodimerization increased in GEO C (Fig. 2C). These results suggest that EGFR may compensate for ErbB2 loss in transphosphorylating ErbB3 through heterodimerization in antibody-transfected cells. Consequently, EGFR expression and activation were investigated.

**EGFR Expression and Activation**—Expression and activation of EGFR were low in GEO wild type cells. When GEO cells were transfected with the single chain antibody against ErbB2, EGFR expression and activation increased dramatically (Fig. 3). The activation of EGFR was assayed either by immunoprecipitation using phosphotyrosine antibody 4G10 or by Western blot using an activated EGFR antibody (Y1173) as described under "Materials and Methods." On the other hand, ErbB3 expression as well as ErbB2 remained almost the same in GEO C compared with GEO parental cells (data not shown). These results implied that EGFR compensated for the loss of ErbB2 activation in GEO C through increased expression and activation.

We hypothesized that increased EGFR activation may play a role in GEO C cell survival because our previous studies had shown that ErbB2 was critical to cell survival signaling in GEO cells (40). Treatment of cells with the highly selective EGFR inhibitor AG1478 (51) showed that DNA fragmentation was significantly increased in GEO C cells compared with that of GEO control cells (Fig. 4A). In a separate experiment, different concentrations of anti-EGFR MAb225 treatment showed similar results (Fig. 4B). Western blot results showed that both AG1478 and MAb225 inhibit p-EGFR in GEO and GEO C cells efficiently, but PARP cleavage was increased strongly in GEO C cells as AG1478 or MAb225 concentrations increased (Fig. 4). These results indicated that increased EGFR activation contributed to maintenance of cell survival in GEO C cells. Furthermore, this implies a shift of roles between ErbB2 and EGFR in supporting survival after activated ErbB2 was eliminated in the transfected cells.

**Trapping ErbB2 in the ER Does Not Activate ER Stress Signals**—Transfection of ScFv5R retains ErbB2 protein in the ER. This could generate an increased work load in this compartment, which might in turn lead to an activation of ER stress signaling pathways. When stress is applied to this compartment in a prolonged manner, these signaling pathways have been shown to activate proapoptotic processes (52). These could be interpreted as a direct effect of the reduction of ErbB2 levels at the plasma membrane. To test whether the ER stress signaling pathways are affected by the expression of the single chain antibody, we performed analyses of the transcription of an ER stress-specific target, BiP, and determined the phosphorylation status of the translation initiation factor eIF2α. BiP is an ER chaperone that is regulated by ER signaling kinase IRE1 and is induced upon ER stress (52, 53). ER stress will also increase the activity of another ER-resident kinase, PERK. PERK phosphorylates eIF2α on serine 51, inhibiting translation of messenger RNA (54). To analyze BiP transcription, we extracted RNA from GEO and GEO C cells, then performed reverse transcription-PCR and quantitative PCR. The results showed that there are no differences between GEO and GEO C in BiP transcription in both confluent and quiescent cells (Fig. 5A). We also performed Western blot using a specific antibody that recognizes eIF2α phosphorylated at Ser-51. Confluent GEO and GEO C cells have same level of p-eIF2α (Ser-51) as well as total eIF2α (Fig. 5B). Quiescent cells have the same level of total eIF2α, and phosphorylation of eIF2α was undetectable in both cell types (data not shown). These results indicated that neither ER stress signaling pathway is activated by the expression of the ScFv5R antibody against ErbB2. Taken together, the results above are consistent with earlier work done by others (40, 46), indicating that the single chain antibody against ErbB2 successfully traps ErbB2 in the ER, blocks its activity, and results in reduced heterodimerization of ErbB2 and ErbB3.

**The ScFv5R-transfected Cells Show Greater Susceptibility to Apoptotic Stress than GEO Cells**—Because loss of ErbB2 function in the ScFv5R-transfected GEO cells altered cell signaling, biological effects were likely to be triggered as well. We showed previously that the phosphatidylinositol 3-kinase and ERK pathways were linked to ErbB2/ErbB3 in GEO cells, and both pathways were implicated in protection from cell death (39). Thus, we hypothesized that the loss of ErbB2 signaling in ScFv5R antibody-transfected cells might make the cells more susceptible to cell death by stress induced through growth.

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**Fig. 5.** ER stress signals not activated by trapping ErbB2 in the ER. A, RNA was collected from confluent (CONF.) or quiescent day 5 (QUI.) GEO and GEO C cells, and a two-step reverse transcription-PCR procedure was performed. Shown are linear data normalized to the level of confluent GEO (GEO CONF.) cells from BiP gene quantitative PCR (mean ± S.E.; n = 3). A glyceraldehyde-3-phosphate dehydrogenase-JOE probe was used as endogenous control. B, confluent GEO and GEO C cells were harvested, and lysates were collected. Equivalent amounts of the cell extracts were analyzed by SDS-PAGE using phospho-eIF2α (Ser-51) antibody and the total eIF2α antibody. Anti-Hsp70 was used as loading control in this experiment. Densitometric analysis of the phospho-eIF2α (Ser-51) Western blot data normalized to GEO cells is shown graphically (units on the y axis are arbitrary units representing density). Error bars stand for S.D. Data shown are representative of three independent experiments.
factor deprivation. Cells were harvested after various periods of deprivation as described under “Materials and Methods” and evaluated for evidence of DNA fragmentation (DNA laddering) and PARP cleavage (55–57). An “apoptotic ladder” of DNA fragments was first observed 3 days after removal of growth factors (Qd3), ladderling continued to increase until Qd5. At each time point, the amount of DNA laddering from GEO C cells was increased relative to that from GEO control cells (Fig. 6A), thus indicating that the ScFv5R-transfected cells had a greater propensity for apoptosis in response to growth factor deprivation stress. This was confirmed by PARP cleavage. PARP is one of the earliest proteins targeted for a specific cleavage to the signature 85-kDa fragment generated during apoptosis (56). The 85-kDa apoptotic fragments in Fig. 7A showed that GEO C displayed more apoptosis than GEO. Addition of tetracycline reverses apoptosis in transfected cells back to the level of control cells (Figs. 6B and 7B). Apoptosis was also determined by flow cytometry analysis (Fig. 8). Apoptosis at Qd3 by transfected cells was severalfold higher than that of GEO- or GEO C tetracycline-treated cells, whereas the levels were 3–4-fold higher at Qd4 and Qd5. These results supported the DNA laddering and PARP cleavage results, indicating that blockade of ErbB2 signaling resulted in sensitization to stress-induced apoptosis.

We utilized AG879, a highly selective phosphorylation inhibitor for ErbB2, to determine acute effects on survival signaling resulting from inhibition of ErbB2 function (46, 51). Quiescent GEO cells were treated with AG879, and phosphorylation of ErbB2 was examined. AG879 inhibited ErbB2 phosphorylation in a concentration-dependent manner (Fig. 9A), and as activation of ErbB2 was reduced, PARP cleavage increased (Fig. 9B). Caspase-3 is one of the key executors of apoptosis and is responsible for the proteolytic cleavage of the PARP (58). Activation of caspase-3 requires cleavage of its own subunits (59).

Our results showed that cleavage of caspase-3 increased as the concentration of AG879 increased (Fig. 9C), further indicating that inhibition of ErbB2 phosphorylation by AG879 induces apoptosis, similar to the effects of the ScFv5R single chain antibody on GEO stress-induced apoptosis. We also characterized the effect of AG879 treatment on EGFR phosphorylation. GEO cells were treated with different concentrations of AG879 for 5 h at 37 °C, then the media were changed to fresh SM medium plus AG879 for another 24 h. Although ErbB2 activation was blocked by AG879, EGFR phosphorylation increased in GEO cells (Fig. 9A).

**DISCUSSION**

We reported previously that GEO colon carcinoma cells activate ErbB2 through heregulin-mediated autocrine activity, and ErbB2 is not overexpressed in GEO cells (39). Moreover, we found that heregulin, ErbB2, and ErbB3 were expressed in several human colon cancers, but not overexpressed. The constitutive activation of the normal levels of ErbB2 leads to a highly progressed phenotype in GEO cells similar to that reported previously by ectopic transforming growth factor-α expression in relatively indolent tumor cells derived from early stage malignancies (16). Therefore, constitutively activated ErbB2 in tumors might represent a potential target for receptor-based therapeutic approaches. Thus far, many strategies...
have been developed to target specifically tumor cells expressing ErbB2 oncoprotein. These strategies include chemical inhibitors (61), dominant kinase-negative mutants (38, 62), monoclonal antibodies (63, 64), and antisense gene inhibition (65). However, these strategies have been focused mainly on ErbB2 overexpressing tumor cells.

In this study, we used an alternative approach to accomplish highly selective, targeted ablation of ErbB2 through the introduction of a single chain antibody that traps the ErbB2 protein in the ER thereby inactivating ErbB2 activity. This effect leads to biological changes in the cells. Removal of ErbB2 from the cell surface enhanced the apoptotic response to stress and was associated with reduced xenograft growth by antibody-transfected cells. These findings are in accordance with the in vitro observations by Beerli et al. (40) in ErbB2-transformed fibroblasts; they described the stable inactivation of ErbB2 onco-
programmed cell death and reduced malignancy in vivo, indicating that ErbB2 may be a key factor in sustaining the malignant phenotype in this type of cancer. However, although the ErbB2 antibody-transfected cells showed reduced malignancy, they were still capable of growth both in vitro and in vivo, indicating the ability to develop a compensatory mechanism for loss of ErbB2 activation.

Interestingly, stable removal of ErbB2 kinase activity by receptor trapping not only results in hypersensitivity to apoptosis induced by growth factor deprivation relative to wild type GEO cells, but also increased EGFR activity as well (Fig. 3). GEO cells are of particular interest because of their widespread use as an in vivo model for EGFR antagonists and their combination with cytotoxic drugs such as cisplatin and CPT 11 (69, 70). The importance of GEO as an in vivo model for EGFR antagonists stems from its xenograft growth dependence on autocrine EGFR activation as we described previously (15). Here we demonstrated that loss of functional ErbB2 activity after its retention in the ER or by a selective kinase inhibitor (AG879) leads to increased activity of EGFR. This indicates that EGFR may compensate for the loss of ErbB2 function in GEO C cells. EGFR plays a more important role in supporting survival of GEO C cells as shown in this study. However, we still see important changes such as increased apoptosis in the transfected cells, suggesting that the compensation is not completely effective. Because many cells demonstrate multiple cell survival mechanisms, the pathway utilized by EGFR might be completely different from that of ErbB2 and contributing a second cell survival pathway with a different mechanism from that of ErbB2 signaling.

In summary, activation of specific ErbB2 signal transduction pathways leads to transformation and malignancy of GEO cells at normal expression levels. EGFR activation increased when ErbB2 activity was removed by the ScFv5R transfection or by the kinase inhibitor AG879. Blocking ErbB2 function leads to alterations of the signaling pathways and modulation of tumorigenicity as well as EGFR compensation. Moreover, this compensation by EGFR can occur within hours of ErbB2 inhibition as shown by treatment of GEO cells with AG879. The emergence of this compensatory mechanism, both acutely and long term, has implications for cancer therapies directed at the ErbB2 receptor and may also extend to compensatory responses to other ErbB family members and other receptors. These implications support the potential value of the development of combination targeted therapies encompassing multiple receptors.

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