Epidermal Growth Factor–Independent Transformation of Ba/F3 Cells with Cancer-Derived Epidermal Growth Factor Receptor Mutants Induces Gefitinib-Sensitive Cell Cycle Progression

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Abstract

Epidermal growth factor receptor (EGFR) plays critical roles in many biological processes and in tumorigenesis. Here, we show that two mutated EGFRs found in lung and other malignancies, EGFR-G719S and EGFR-L858R, could transform Ba/F3 cells to interleukin-3 (IL-3)–independent growth, in a ligand-independent manner, an activity associated with the transforming function of other mutated tyrosine kinases. The mutated receptors are autophosphorylated in the absence of IL-3 without EGF stimulation, and their expression led to the constitutive activation of signal transducers and activators of transcription 5, extracellular signal-regulated kinase 1/2 (ERK1/2), ERK5, and AKT. In wild-type EGFR-expressing Ba/F3 cells, the major EGF-mediated signaling pathways were still intact. Gefitinib inhibited the growth of mutant EGFR-transformed Ba/F3 cells. Strikingly, the gefitinib sensitivity of cells expressing the L858R mutant was significantly greater than that of cells expressing the G719S mutant form, suggesting that distinct EGFR mutations may be differentially sensitive to small-molecule inhibitors. Furthermore, our data showed an antiproliferative effect of gefitinib on the EGFR-transformed Ba/F3 cells. Our results provide a model system to study the function of mutated EGFR and the differential effects of pharmacologic EGFR inhibition on the distinct mutant forms of this tyrosine kinase. (Cancer Res 2005; 65(19): 8968-74)

Introduction

Epidermal growth factor receptor (EGFR, HER1, and c-erbB1) is a membrane-bound glycoprotein and belongs to the HER/ERBB growth factor receptor family. EGFR can form either homodimers with other EGFRs or heterodimers with other HER family members, including HER2, HER3, and HER4 (1, 2). Upon binding of its polypeptide ligands, EGFR initiates signaling cascades that regulate cellular processes. The importance of EGFR in development is shown by EGFR knockout mice that have congenital organ defects and die within the first month of birth (2, 3).

The first EGFR somatic mutation characterized was an 801-bp in-frame deletion of the exons 2 to 7 within the extracellular ligand-binding domain of wild-type EGFR, known as EGFRvIII (4–6). It has been shown that expression of EGFRvIII leads to a ligand-independent, constitutive activity (6–8) and can induce tumors in a murine glioma model (9).

Recently, somatic mutations in the kinase domain of EGFR have been discovered in lung adenocarcinomas (10–12). The finding that overexpression and mutations of EGFR occurred in many types of human cancer make EGFR a promising target for anticancer therapy. The specific inhibitors, including gefitinib and erlotinib, which compete with ATP for binding to the kinase domain of EGFR, have been approved for clinical use in non–small cell lung cancer (13). The dramatic responses to gefitinib and erlotinib of lung adenocarcinomas harboring EGFR mutations (10–12), as well as the finding that secondary mutations in the EGFR kinase domain cause resistance to gefitinib (14, 15), indicate that mutated EGFR appears to be their major target. The antiproliferative or apoptotic effects (16–18) of gefitinib have been documented, although the mechanism of its action remained largely unknown, especially in the cells harboring mutations of EGFR.

The major signaling cascades induced by ligand-activated wild-type EGFR include mitogen-activated protein kinase (MAPK) pathways, signal transducers and activators of transcription (STAT) pathways, and the phosphatidylinositol 3-kinase (PI3K) pathway (reviewed in ref. 19). MAPK pathways can be activated in a Ras/Raf-dependent or Ras/Raf-independent manner, converging on extracellular signal-regulated kinase (ERK1/2) and ERK5. STAT activation seems dependent on EGFR tyrosine kinase activity (20–22), whereas PI3K activity leads to phosphorylation and activation of the AKT kinase (23). Studies of cells expressing EGFR mutations in the kinase domain revealed phosphorylation of STAT5 and AKT (16) and Src homology and collagen (Shc; ref. 18). NIH-3T3 cells can be transformed by mutant EGFRs, leading to activation of Shc and STAT3. 6

The growth of the murine bone marrow–derived cell line, Ba/F3, is dependent on the growth factor interleukin-3 (IL-3) but is rendered IL-3 independent by several tyrosine kinase oncogenes, including BCR/ABL (24), FLT3 (25), and TEL/platelet-derived growth factor receptor β (26). For such oncogenes, induction of factor independence of Ba/F3 cells has been closely associated with transforming activity in vitro and in vivo (27).

In this study, we characterized Ba/F3 cells expressing two missense mutations in the kinase domain of EGFR, EGFR-L858R and EGFR-G719S, and showed that these mutants transformed Ba/F3 cells to factor independence in the absence of exogenous EGF.

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Our results provide conclusive evidence of constitutive activation of these mutant receptors and provide a model system to study the effects of EGFR inhibitors and downstream targets. Strikingly, we find a difference in response to gefitinib between the two mutants and show that gefitinib acts by inhibiting cell cycle progression in these cells. This system should prove useful for further studies of EGFR inhibitor action.

**Materials and Methods**

**Reagents and antibodies.** Gefitinib was obtained from AstraZeneca (Waltham, MA). AG1478, EGF, RNase A, propidium iodide, and anti-tubulin antibody (clone DM 1A) were from Sigma (St. Louis, MO). The following antibodies were used: pTyr (PY99), pERK (E4), EGFR (1005), ERK5 (H-300), CDK4(c-22), CDK6 (c-21), Cyclin D2 (c-17), and Cyclin D3 (c-16) from Santa Cruz Biotechnology (Santa Cruz, CA); pAKT (S240/S244), AKT, pERK5 (Thr218/Tyr220), p44/42 MAPK from Cell Signaling (Beverly, MA); p-STAT5 (Tyr694), STAT5 (clone 89), EGFR (clone 13), p27 (clone 57) from BD Transduction Laboratories (Lexington, KY).

**Generation of EGFR-L858R and EGFR-G719S containing expression vectors and transfection into Ba/F3 cells.** Full-length cDNAs of EGFR containing L858R and G719S mutations were generated by mutagenesis using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutated full-length EGFR cDNAs were confirmed by sequencing. The cDNAs of EGFR-L858R, G719S, +/− IL-3, and +/− IL-3 were electroporated into murine Ba/F3 cells (Stratagene, La Jolla, CA), which was then subjected to Western blotting analysis using the specific antibodies indicated.

**Cell culture.** Ba/F3 cells were cultured in RPMI 1640 (Mediatech, Inc., Herndon, VA) with 10% FCS, 10% WEHI3B conditional medium (as a source of IL-3), 100 units/ml penicillin and 100 mg/ml streptomycin, and 1% glutamine. Ba/F3-EGFR-L858R and Ba/F3-EGFR-G719S cells were maintained in the culture medium as its parental Ba/F3 cells, except without IL-3, but with 800 mg/mL G418. Ba/F3-EGFR wild-type cells were cultured in the medium with IL-3 and 800 mg/mL G418. All the cells were maintained in a humidified incubator at 37°C with 5% CO2.

**Western blotting analysis.** Cells were collected and washed once in cold PBS and then lysed in NP40 buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% NP40, and 1× Protein Inhibitor Cocktail Set I (Calbiochem, San Diego, CA)]. After incubation in ice for 30 minutes, the lysates were centrifuged at 14,000 × g for 15 minutes, and the supernatant was transferred into a new tube. The protein concentration of the supernatant was detected by the Bio-Rad protein assay kit (Richmond, CA) with 10% FCS, 5% CO2, 10% WEHI3B conditional medium (as a source of IL-3), 100 units/ml penicillin and 100 mg/ml streptomycin, and 1% glutamine. Ba/F3-EGFR-L858R and Ba/F3-EGFR-G719S cells were maintained in the culture medium as its parental Ba/F3 cells, except without IL-3, but with 800 mg/mL G418. Ba/F3-EGFR wild-type cells were cultured in the medium with IL-3 and 800 mg/mL G418. All the cells were maintained in a humidified incubator at 37°C with 5% CO2.

**Figure 1.** IL-3-independent proliferation of Ba/F3 cells expressing mutant EGFR, associated with constitutive EGFR autophosphorylation. A, Ba/F3 cells stably expressing EGFR-L858R or EGFR-G719S or pClneo empty vector were seeded at a density of 1 × 105/mL and grown in the presence or absence of IL-3 (labeled as G719S, +/− IL-3; L858R, +/− IL-3; and pClneo, +/− IL-3). Cells were collected at the time indicated and counted after staining with trypan blue. Points, means of two independent experiments; bars, SD. B, as controls, Ba/F3 cells transfected with empty vector (pClneo) or with wild-type EGFR were grown and analyzed as above in the presence or absence of EGF or IL-3 (labeled as WT, +/− IL-3+/- EGF; pClneo, +/− IL-3). C, immunoblots with anti-EGFR antibody 1005 (top) and antiphosphotyrosine antibody pY99 (bottom) from lysates of Ba/F3 cells transfected with vector (pClneo), EGFR-L858R, or EGFR-G719S. Lanes 1 and 4, pooled populations stably expressing EGFR-L858R and EGFR-G719S, respectively; lanes 2, 3, 5, and 6, clones derived from the pooled population. D, immunoprecipitation with control IgG (Con IgG) or anti-EGFR antibodies from lysates of Ba/F3 cells expressing EGFR-L858R or EGFR-G719S, as above, followed by immunoblotting with anti-EGFR antibody 1005 (top) and antiphosphotyrosine antibody pY99 (bottom). Representative of two independent experiments.
Immunoprecipitation and immunoblotting. Immunoprecipitations were done using 1,000 to 1,500 mg of total protein as starting materials following a protocol suggested by Santa Cruz Biotechnology. Briefly, the reaction volumes were equalized using NP40 lysis buffer. The samples were precleared with control IgG followed by incubation with primary antibody and corresponding protein-agarose. At the end of the incubation, the beads were washed four times with TNN buffer [40 mmol/L Tris-HCl (pH 8.0), 10 mmol/L NaCl, 0.5 % NP40]. After the final washing, the pelleted beads were resuspended in 1 L Laemmli sample buffer and the supernatant was subjected to Western blotting analysis following the standard procedures.

Fluorescence-activated cell sorting analysis. Cells were collected and fixed in 40% ethanol for at least 1 hour (or until ready for the experiment) at 4°C. The fixed cells were treated with 0.5 mL of 500 mg/mL RNase A for 45 minutes at 37°C and stained with 69 mmol/L propidium iodide (in 38 mM sodium citrate) for at least 30 minutes at room temperature in the dark. The stained cells were then analyzed for DNA content in a Becton Dickinson fluorescence-activated cell sorter using both ModFit (Verity Software House, Topsham, ME) and CellQuest (Becton Dickinson, San Jose, CA) programs.

Results

Interleukin-3-independent growth of Ba/F3-EGFR-L858R and Ba/F3-EGFR-G719S cells and constitutive autophosphorylation of the mutated receptors. To determine the transforming potential, pClneo expression vector (with G418-selective marker) containing EGFR, EGFR-L858R, EGFR-G719S, and empty pClneo were each transfected into IL-3-dependent Ba/F3 cells. After selection in G418, IL-3 was withdrawn from the medium. Whereas cells transfected with either pClneo or pClneo-EGFR failed to grow, polyclonal populations of cells transfected with pClneo-EGFR-G719S or pClneo-EGFR-L858R were readily obtained and able to proliferate, with a growth rate similar to that in the presence of IL-3 and comparable with those of Ba/F3 cells transfected with pClneo alone grown in the presence of IL-3 (Fig. 1A). In contrast, the expression of wild-type EGFR did not permit factor-independent growth of Ba/F3 cells (Fig. 1B), even at the levels higher than those for some IL-3-independent, mutant EGFR-expressing cells (data not shown). In the absence of IL-3, some slight growth of Ba/F3-EGFR cells was observed upon EGF stimulation but much slower than for cells grown in the presence of IL-3 (Fig. 1B), which agree with previous findings (28–30).

To determine whether IL-3-independent growth was associated with constitutive activation of the mutated receptors without EGF stimulation, we analyzed the tyrosine phosphorylation status of the receptors in both polyclonal Ba/F3 populations and cloned lines. Immunoblotting of cell lysates revealed a tyrosine-phosphorylated protein with a molecular weight corresponding to EGFR (Fig. 1C). Immunoprecipitation of EGFR
followed by immunoblotting with antiphosphotyrosine antibodies confirms the identity of this tyrosine-phosphorylated protein as EGFR (Fig. 1D).

These results indicated that the L858R and G719S mutations of EGFRs have transforming potential and that these mutated receptors are constitutively autophosphorylated.

**Differential growth inhibition of epidermal growth factor receptor mutant-expressing Ba/F3 cells by gefitinib.** We next examined the effects of two EGFR-specific inhibitors, AG1478 and gefitinib, on the growth of Ba/F3-EGFR-L858R and Ba/F3-EGFR-G719S cells. Figure 2A shows that both drugs could inhibit the growth of Ba/F3 cells expressing either mutation in the absence of IL-3; whereas in the presence of IL-3, the cells were still able to proliferate at concentrations as high as 300 nmol/L for either drug. The EGFR inhibitors did not inhibit the growth of FLT3-N841I-transformed Ba/F3 cells (25) in both the presence and absence of IL-3 at concentrations up to 3,000 nmol/L.

Strikingly, gefitinib seemed to inhibit growth of cells expressing the two EGFR mutants to different extents. IC50 for AG1478 is about 8 nmol/L in Ba/F3 cells expressing EGFR-L858R and about 17 nmol/L in EGFR-G719S cells, whereas IC50 for gefitinib is about 20 nmol/L in EGFR-L858R-expressing cells and about 140 nmol/L in EGFR-G719S cells. Thus, the EGFR-G719S mutant seems clearly less sensitive to gefitinib.

To examine the effect of gefitinib on mutant EGFR kinase activity, we used receptor autophosphorylation as a marker. Figure 2B shows that inhibition in the absence of IL-3, gefitinib was able to reduce the autophosphorylation levels of mutant EGFRs in a dose-dependent manner, which indicates that in the absence of IL-3, the growth inhibition observed in Fig. 2A is due to the inhibition of the mutant receptors; whereas in the presence of IL-3, the growth of the host cells was not controlled by the mutant receptors. The reason that inhibition of autophosphorylation of EGFR-G719S by gefitinib seems more effective in the presence of IL-3 is currently unknown. Figure 2B also shows that, in the absence of IL-3, the inhibition of autophosphorylation of EGFR-G719S required higher concentration of gefitinib compared with that of EGFR-L858R, indicating that the differential growth inhibition seen in Fig. 2A may be due to differential inactivation of the mutant receptors.

**Constitutive activation of downstream pathways by the mutated epidermal growth factor receptors in Ba/F3 cells.** To explore the downstream targets of EGFR-G719S and EGFR-L858R, the phosphorylation of several known effectors of the EGFR signaling pathways were analyzed. For cells expressing the EGFR mutants, STAT5, ERK1/2, AKT, and ERK5, are all phosphorylated in the absence of IL-3, without exogenous EGF stimulation (Fig. 3A, lanes 1 and 7). Gefitinib was able to reduce the phosphorylation of each of these downstream effectors (Fig. 3A, lanes 2, 3, 8, and 9); whereas pAkt was still detectable at the highest gefitinib dose, the levels were clearly reduced. In contrast, the activation of these downstream effectors by FLT3-N841I was not affected by gefitinib (Fig. 3A, lanes 13 and 14). IL-3 treatment of cells transfected with vector alone led to the phosphorylation of STAT5 but not the other EGFR effectors (Fig. 3A, lanes 17 and 18). Correspondingly, in IL-3-supplemented cells expressing EGFR mutants, STAT5 phosphorylation becomes resistant to gefitinib, whereas the other downstream effectors remain sensitive (Fig. 3A, lanes 4-6, 10-12). This result indicates that the phosphorylation of STAT5 in the presence of IL-3 was not controlled by the mutated EGFRs but by other pathways.
probably through IL-3 pathway, because IL-3 is a strong activator of STAT5 (31, 32).

To determine whether serum in the medium may contribute to the observed constitutive phosphorylation of mutant EGFR and its downstream effectors, even in the absence of exogenous EGF, we also examined their phosphorylation status in the presence and absence of serum. The mutated receptors were autophosphorylated to a similar level after starvation from serum for 24 hours compared with those growing in the presence of serum, which was also the case for the phosphorylation of STAT5, AKT, and ERK5 (Fig. 3B). Phosphorylation of ERK1/2 was not affected by serum starvation in cells expressing EGFR-L858R and remained detectable after serum starvation of cells expressing EGFR-G719S. In contrast, the phosphorylation of ERK1/2, as well as other tested kinases, was diminished significantly upon serum starvation in the cells transfected with pClneo alone (Fig. 3B). These results showed that the EGFR mutations were able to confer constitutive kinase activities to the EGFRs and to activate major downstream targets, in the absence of EGF or any other EGFR ligand.

Epidermal growth factor–mediated signaling in wild-type and mutated epidermal growth factor receptor–expressing cells. Given the constitutive activation of the mutant EGFRs, we wished to determine whether the signaling pathways downstream of the wild-type receptor could be activated by EGF in Ba/F3 cells and whether the mutant receptors could be superactivated by addition of exogenous EGF. We first examined the response of wild-type EGFR to EGF stimulation. Upon EGF stimulation, wild-type EGFR was autophosphorylated, which could be blocked by gefitinib as examined by either immunoblotting (Fig. 4A) or immunoprecipitation/immunoblot analysis (data not shown). Furthermore, EGF was able to induce phosphorylations of STAT5, ERK1/2, AKT, and ERK5 in wild-type EGFR-expressing Ba/F3 cells (Fig. 4A, lanes 5-7). As for EGF-mediated receptor autophosphorylation, gefitinib inhibited phosphorylation of these kinases, indicating that they are downstream targets of the EGF-activated wild-type EGFR (Fig. 4A, lanes 6 and 7). These results indicate that the EGF-mediated major signaling pathways of wild-type EGFR found in other systems are all intact in Ba/F3 cell systems.

The mutant receptors were found to respond further to the addition of EGF. Figure 4B shows that in cells expressing either G719S or L858R mutant, the autophosphorylation of mutated receptors and the phosphorylation of STAT5 were significantly induced by EGF. EGF stimulation had only subtle effects on phosphorylation of ERK5 and ERK1/2 and had a more potent effect on the phosphorylation of AKT in G719S-expressing cells than that in L858R cells. In each case, gefitinib was able to block EGF-mediated signaling, indicating that these signaling pathways were regulated through mutant EGFR itself (Fig. 4B, lanes 3 and 6).

Antiproliferative effects of gefitinib on Ba/F3 cells expressing mutated epidermal growth factor receptors. Dependent on the genetic and physiologic background of the host cells, gefitinib could have either a proapoptotic effect or an antiproliferative effect (33). To examine the cell cycle effect of gefitinib, the EGFR-L858R- and EGFR-G719S-expressing Ba/F3 cells were treated with 0.1 μmol/L (for EGFR-L858R cells) or 0.5 μmol/L (for EGFR-G719S cells) of the drug, and at the time point indicated, the cells were collected for both protein extraction and fluorescence-activated cell sorting analysis. Figure 5A shows that gefitinib induced G1 cell cycle arrest in the G719S and L858R cells starting as early as 12 hours. After 72 hours of treatment, most of the cells were blocked in the G1 phase, indicating an antiproliferative effect of gefitinib on the cells.
To determine the potential molecular mechanism of gefitinib-induced G₁ cell cycle arrest in these cells, the cell cycle regulators important for initiation of G₁-to-S phase transition were analyzed. Figure 5B shows that the levels of cyclin D2 and D3 went down as early as 3 hours after exposure to drug in the G719S and L858R cells but rose back after 24 hours of treatment. The level of CDK4 also went down in the EGFR mutant-expressing cells and reached the lowest level 24 hours after treatment. In L858R cells, CDK4 level started to decrease at about 3 hours after treatment, and at 12 hours, there was almost no detectable CDK4. In G719S cells, the level of CDK4 decreased slightly after 3 hours of treatment and declined more sharply after 6 hours. The level of CDK6 was not altered by gefitinib. p27 levels showed the most delayed response, starting to increase 6 hours after gefitinib treatment and reaching the highest level at 24 hours. The results indicated that geftinib-induced G₁ cell cycle arrest was preceded by down-regulation of the levels of D-type cyclins and CDK4.

Discussion

In this report, we showed that two clinically relevant point mutations, L858R and G719S, within the kinase domain of EGFR, represent novel ligand-independent activating mutations and confer constitutive kinase activity and IL-3-independent proliferation to murine growth factor–dependent Ba/F3 cells. The abilities of the mutated receptors to activate ERK5, ERK1/2, STAT5, and AKT constitutively in the absence of EGF are consistent with their oncogenic potential.

Ba/F3 cells are especially suitable for studies of mutant EGFR activation, as these cells do not express endogenous EGFR or other members of the ErbB family (28, 29). In particular, the use of this system has allowed the identification of the ligand-independent activation of EGFR induced by single missense point mutations in the kinase domain. Transformation and ligand-independent activation by mutant EGFR have also been observed in NIH-3T3 cells.

The specific transforming potential of mutant EGFRs relative to wild-type EGFR was apparent even at early stages of selection. In contrast to Ba/F3 cells expressing EGFR-L858R or EGFR-G719S, which survived and started to proliferate in the absence of IL-3, cells expressing wild-type EGFR failed to grow and died out eventually, despite similar expression levels of mutant and wild-type EGFR at this early stage.

Whereas L858R and G719S mutations both conferred ligand-independent activation to EGFR, their sensitivities to gefitinib are different. L858R is almost 10-fold more sensitive to the drug. Such difference in sensitivity may be due to the fact that G719S is located in the ATP binding loop (p-loop) and gefitinib is a competitor of ATP. Due to its small side chain that could fit into niches where no other amino acid could fit in, glycine plays a unique role in the conformation of many proteins. Changing from glycine to serine may disrupt the local fine structure and induce a less favorable conformation for gefitinib to bind.

The antiproliferative and proapoptotic effects of gefitinib in a wide range of tumors have been well documented (34–36). Its antiproliferative effect has been reported due to G₁ cell cycle arrest via up-regulation of p27 (37, 38) and down-regulation of D-type cyclins and CDK4/6 (38). Whereas the proapoptotic effect of gefitinib has been noticed (16, 17), its antiproliferative effect on cells expressing mutant EGFRs has not been reported. Our finding that in EGFR-L858R- and EGFR-G719S-expressing Ba/F3
cells gefitinib induced G1 cell cycle arrest, preceded by downregulation of D-type cyclins and CDK4, adds a new role and potential new mechanism for gefitinib action.

The antiproliferative versus proapoptotic activity of gefitinib in different systems may depend on the genetic background and physiologic conditions of host cells. In our hand, when used at a concentration 5 to 10 times the IC50, gefitinib induced a G1 cell cycle arrest, but no apoptotic cells were detected for over 72 hours for both mutated receptors, whereas in lung cancer cell lines harboring L858R mutation gefitinib induced apoptosis (16, 17). It will be interesting to find out whether higher concentrations of gefitinib could induce apoptosis in Ba/F3 cells. A comparison of signaling pathways in EGFR mutant-expressing Ba/F3 cells and in lung carcinoma cells harboring the EGFR-L858R mutation may aid in dissecting antiproliferative effects from proapoptotic effects of gefitinib, an important issue when the drug is used in combination with chemotherapy (33).

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