Rat Prolactinoma Cell Growth Regulation by Epidermal Growth Factor Receptor Ligands

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Abstract

Epidermal growth factor (EGF) regulates pituitary development, hormone synthesis, and cell proliferation. Although ErbB receptor family members are expressed in pituitary tumors, the effects of EGF signaling on pituitary tumors are not known. Immunoprecipitation and Western blot confirmed EGF receptor (EGFR) and p185<sup>new</sup> protein expression in GH3 lacto-somatotroph but not in adrenocorticotropic hormone–secreting AT20 pituitary tumor cells. EGF (5 nmol/L) selectively enhanced baseline (−4-fold) and serum-induced (>6-fold) prolactin (PRL) mRNA levels, whereas gefitinib, an EGFR antagonist, suppressed serum-induced cell proliferation and Ptg1 expression, blocked PRL gene expression, and reversed EGF-mediated somatotroph-lactotroph phenotype switching. Downstream EGFR signaling by ERK, but not phosphoinositide-3-kinase or protein kinase C, mediated the gefitinib response. Tumors in athymic mice implanted s.c. with GH3 cells resulted in weight gain accompanied by increased serum PRL, growth hormone, and insulin growth factor 1. Gefitinib decreased tumor volumes and peripheral hormone levels by −30% and restored normal mouse body weight patterns. Mice treated with gefitinib exhibited decreased tumor tissue ERK1/2 phosphorylation and downregulated tumor PRL and Ptg1 mRNA abundance. These results show that EGFR inhibition controls tumor growth and PRL secretion in experimental lacto-somatotroph tumors. EGFR inhibitors could therefore be useful for the control of PRL secretion and tumor load in prolactinomas resistant to dopaminergic treatment, or for those prolactinomas undergoing rare malignant transformation. [Cancer Res 2008;68(15):6377–86]

Introduction

Pituitary adenomas account for ~15% of primary intracranial neoplasms and are discovered in up to 25% of unselected autopsy specimens. Despite their benign nature, tumor growth may lead to critical local compressive symptoms and altered hormone secretion resulting in distinct endocrine syndromes, depending on the tumor cell type (1). Pituitary adenomas arise as clonal expansions of mutated somatic cells, but the sequence of initial transforming events are unclear and occur on a background of chromosomal instability, epigenetic alterations, and mutations. Given the rich vascularization of the gland, and the tight hypothalamic control of hormone secretion, alterations within the pituitary microenvironment including paracrine or autocrine dysregulation and growth factor disruption may be permissive for accelerated growth (1).

Pituitary tumors arising from the lacto-somatotroph cell lineage, which secrete growth hormone (GH) and/or prolactin (PRL), usually respond to medical treatment with dopamine agonists and/or somatostatin analogues (1–3). Medical therapy may however be limited due to dopamine or somatostatin receptor resistance (2, 3), or drug intolerance. For adrenocorticotropic hormone–secreting and clinically nonfunctioning pituitary adenomas, no effective drug therapies currently exist (4, 5). The success of trans-sphenoidal pituitary surgery is highly dependent on the experience and expertise of the surgeon, and the size and type of tumor. Radiotherapy, used after failed trans-sphenoidal surgery and/or medical therapy, is associated with relatively high complication rates (2–6). Treatment alternatives are particularly required for recurring invasive macroadenomas and for infrequently encountered but aggressive pituitary carcinomas which respond poorly to currently available therapies (7, 8).

Aberrant ErbB receptor activation is implicated in several human cancers, leading to the development of novel targeted therapeutics, including monoclonal antibodies and small compound tyrosine kinase inhibitors (9). Although ErbB receptors are expressed in pituitary adenomas (10, 11), and are particularly abundant in very rarely encountered pituitary carcinomas (12, 13), the effects of epidermal growth factor (EGF) receptor (EGFR) signaling on pituitary tumors are unknown. EGFR inhibition in hormonally inactive pituitary folliculostellate cells resulted in reduced Ptg expression (14), a marker for pituitary tumor growth implicated in tumorigenesis and paracrine regulation (15).

Here, we characterize EGFR-mediated pituitary signaling and report the effects of EGFR blockade on hormonally active pituitary tumor cells in vitro and in vivo. The results indicate that EGFR blockade controls experimental pituitary tumor growth and hormone secretion. EGFR inhibition could therefore serve as a molecular target for treating patients unresponsive to currently available forms of medical therapy.

Materials and Methods

Materials. DMEM and RPMI media, fetal bovine serum (FBS), penicillin, streptomycin, and amphotericin B were purchased from Invitrogen. EGF and NRG1-β1/HRG1-β1 were from Sigma. GF109203X was purchased from Biomol. Y-27632, LY294002, PP2, JAK inhibitor I, SB203580, and JNK inhibitor I were from Calbiochem. U0126 was from Promega and gefitinib (Iressa) was purchased from Biaffin GmbH & Co.

Cell culture. AtT20 mouse corticotroph, GH3 rat lacto-somatotroph, and MMQ lactotroph cells were purchased from American Type Culture Collection. The B104-1-1 cell line was a kind gift from Dr. M.I. Greene (Department of Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA). The B104-1-1 cell line was a kind gift from Dr. M.I. Greene.
Medicine, Philadelphia, PA). After synchronization by serum starvation (GH3 cells in medium containing 0.2% bovine serum albumin for ~24 h; AtT20 in 1% FBS medium for ~16 h), cells were plated onto 100 mm dishes (~1.5 x 10^6 cell density) or six-well plates (~0.5 x 10^6 cell density), treatment agents were added with fresh serum-depleted medium (0.2% bovine serum albumin), and samples collected at the indicated times.

**Templates for probes and Northern blot analysis.** Probes for murine POMC, Pttx1, rat GH and PRL were generated as described (16–18). The β-actin probe was a 1.06 kb fragment of the mouse β-actin gene (Amgen). RNA extraction was performed using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. For Northern blot analysis, 10 to 20 μg of total RNA was electrophoresed on a 1% agarose, 4% formaldehyde gel, transferred to a Hybond-N+ membrane (Amersham) and UV cross-linked. Probes were labeled with (α-32P)CTP using the Prime-It random primer labeling kit (Stratagen). Micro Bio-Spin chromatography columns (Bio-Rad) were used to purify probes. Membrane prehybridization and hybridization were performed using QuickHyb Solution (Stratagen) and then exposed to Hyperfilm MP (Amersham) for 1 to 4 days at ~70°C.

**Quantitative PCR.** Total RNA was extracted with Trizol reagent (Invitrogen) according to the instructions of the manufacturer. The amount and the integrity of RNA were assessed by measurement of absorbance at 260 and 280 nm. Before processing, RNA samples were treated with DNase I (Amplification Grade; Invitrogen) to eliminate genomic DNA contamination. Total RNA was reverse-transcribed into first-strand cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Quantitative PCR (Q-PCR) reactions were carried out in the Q5 Multi-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) as previously described (19). Primer sequences (Invitrogen) for rat vascular endothelial growth factor-A (VEGF-A) forward, 5′-TCCCTGGTGCCTCTCATG-3′; reverse, 5′-TGCTGTGTGGATGTTGTTG-3′; rat Pttx1 forward, 5′-CATAGGGCTCTGTCCTGTTG-3′; reverse, 5′-GGCATGAGAAAGGCTGGAAG-3′. Primer sequences for rat GH, PRL, and β-actin have been described previously (19).

**Immunoprecipitation and Western blotting.** After the completion of treatments, the cells were placed on ice and washed with cold PBS. For whole cell protein extraction, cells were lysed in 150 μl of radioimmunoprecipitation assay buffer (Sigma) containing complete protease inhibitor cocktail tablets (Roche Molecular Biochemicals). Lysates were centrifuged at 13,000 x g for 20 min at 4°C and protein concentrations determined by Bradford’s method (Bio-Rad).

Immunoprecipitation with rabbit polyclonal anti-EGFR (3 μg, ab2430; Abcam), polyclonal anti-Neu (C-18, sc-294), and anti-ErbB3 (sc-285, 2 μg; Santa Cruz Biotechnology) was performed with an immunoprecipitation kit (Roche Molecular Biochemicals) according to the instructions of the manufacturer. Cells cultured in 100 mm dishes were lysed in 1 ml of lysis buffer-1 containing protease and phosphatase inhibitor cocktail tablets (Roche Molecular Biochemicals). Preclearing was performed with agaroase G beads (50 μl) overnight at 4°C. Immunoprecipitation with appropriate antibody titers was performed for 1 h prior to the addition of agaroase G beads (50 μl) overnight at 4°C. After extensive washes (twice in wash buffer-1, twice in wash buffer-2, and once in wash buffer-3 for 20 min each), samples were resuspended in 30 μl of sample buffer (Invitrogen).

Immunoprecipitation with monoclonal antibody 7.16.4 (ref. 20; 3 μg: a kind gift from Dr. M.I. Greene) which reacts specifically with rat p185 molecules was performed in cells lysed with modified radioimmunoprecipitation assay buffer (1% Triton X-100, 1% deoxycholate, 0.1% Na2OAc, 0.15 mol/L NaCl, 0.01 mol/L sodium phosphate (pH 7.4), 2 mol/L EDTA, 10 mmol/L sodium PPI, 400 μmol/L sodium orthovanadate) containing complete protease inhibitor cocktail tablets (Roche Molecular Biochemicals). Preclearing was performed with A/G Plus-Agarose beads (20 μl; Sigma) overnight at 4°C. Immunoprecipitation with appropriate antibody titers was performed for 1 h prior to the addition of A/G Plus-Agarose beads (20 μl) overnight at 4°C. Immunoprecipitates were washed six times in washing buffer and resuspended in SDS sample buffer (pH 6.8) as previously described (21).

Western blot analysis was performed according to the guidelines of NuPAGE electrophoresis system protocol (Invitrogen). In brief, whole cell lysates (~50 μg protein per lane) or immunoprecipitation samples were heated for 5 min at 100°C, respectively. Proteins were separated on 4% to 12% NuPAGE Bis-Tris gels and electrotransferred for 1 h to polyvinylidene difluoride (Invitrogen). Membranes were blocked for 1 h in 2% nonfat dry milk (or 5% bovine serum albumin) in TBS-T buffer, and incubated overnight with primary antibody. The following primary antibodies were used: mouse anti-pERK1/2, rabbit anti-ERK1/2 (1:200; Santa Cruz Biotechnology), mouse monoclonal anti-pY799 (PY99), rabbit polyclonal anti-EGFR (sc-01), anti-Neu, anti-ErbB3 (1:200; Santa Cruz Biotechnology), anti-ppDK1 (1:1,000; Cell Signaling), and mouse anti–β-actin (1:1,000; Sigma). After washing with TBS-T, membranes were incubated with peroxidase-conjugated secondary antibody for 1.5 h (2% nonfat dry milk or 5% bovine serum albumin in TBS-T buffer). Blots were washed and hybridization signals measured by enhanced chemiluminescence detection system (Amersham).

**Flow cytometric cell cycle analysis.** Treatments were added after synchronization in fresh serum-depleted medium and samples collected at the indicated times. Cells were washed and fixed in 50% ice-cold ethanol and cell cycle analysis was performed as previously described (14).

**In vivo experiments.** Female athymic NCR-NU mice (8–10 weeks of age) were purchased from Taconic Farms, Inc., and maintained in a laminar airflow unit under aseptic conditions. The research protocol was approved and the care and treatment of experimental animals were in accordance with institutional guidelines. Mice were fed with a commercial pelleted diet and tap water ad libitum, and were allowed to acclimatize for 1 week. For s.c. injections, rat GH3 cells (10^6 cells/0.2 mL of suspension) were injected in both flanks of each mouse. When average tumor volumes reached 3 to 5 mm in diameter (day 1), mice were stratified by tumor volume into two groups of 12 mice (16 tumors per group). Although all 24 mice received two-sided injections of GH3 cells, in some cases, tumor growth was not observed, so the groups were formed to contain equal numbers of mice harboring one (8 mice per group) or two tumors (4 mice per group). Vehicle solution (1% Tween 80; 200 μL) versus gefitinib (125 mg/kg) were administered via oral gavage daily (Monday to Friday) for a total of 14 weekday doses over 18 days. The maximum tolerated dose (150 mg/kg) of gefitinib in NCR-NU mice has been determined previously (with similar administration schedules; refs. 22, 23). A group of mice (n = 12) with no injection of GH3 cells received vehicle solution per oral gavage daily to establish baseline values. Every 3 to 4 days, mice were weighed and tumor volumes measured with a caliper and calculated using the formula, π/6 x large diameter x small diameter2. On the last treatment day (day 18), within ~3 h of drug administration, after determination of body weight and tumor volume, mice were euthanized; cardiac blood collected with 25-gauge syringes (Becton Dickinson) and primary tumors excised and weighed. Fragments of each tumor were fixed in formalin and embedded in paraffin for immunohistochemical staining, preserved in RNAlater solution (Qiagen) for subsequent RNA extraction and frozen in liquid nitrogen for subsequent protein extraction.

**Immunofluorescence.** Slides containing tissue sections were baked for 20 min at 60°C, washed in xylene, ethanol gradient (100%, 95%, 85%, and 75%), and then double-distilled water. For detection of EGFR expression, slides were placed into boiling EDTA (1 mmol/L; pH 8) and incubated in a steamer for 15 min (Black and Decker). Slides were blocked in 5% goat serum and then incubated with primary antibody overnight at 4°C. The following antibodies were used: rabbit polyclonal anti-EGFR (1:50; ab2430; Abcam), guinea pig polyclonal anti-PRL (1:200; courtesy of Dr. A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). Following washes, slides were incubated with Alexa Fluor goat anti-rabbit 488 (H+L) secondary antibody (1:500; Invitrogen) for 1 h at room temperature. Nuclei were stained using 1,500 Topro-3 iodide 1 mmol/L solution (1:250 in PBS, Molecular Probes, Inc.) for 15 min at room temperature, afterwards, slides were mounted with Prolong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (Molecular Probes, Inc). Confocal microscope images were obtained using a TCS-SP confocal scanner (Leica Microsystems). In order to detect contributions of autofluorescence in these paraffin-embedded tissues, a spectral imaging approach was used. The confocal spectrophotometer was set to detect specific FITC
fluorescence in the range of 505 to 540 nm. A second channel detecting autofluorescence with wavelengths from 560 to 600 nm was used. Both channels were color-coded and merged. Green color represents specific fluorescence from FITC and red color represents autofluorescence. The staining was strong and autofluorescence was very low in comparison to fluorescence from FITC and red color represents autofluorescence. The staining was strong and autofluorescence was very low in comparison to fluorescence from FITC.

Hormone assays. RIA for rat GH and PRL were performed in duplicate, using reagents provided by the National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA. Iodination of GH and PRL (5 μg) with iodine-125 (500 μCi; Perkin-Elmer Life & Analytical Sciences) mixed with 0.1 μg of Iodo-Gen (Pierce) was performed using 10 ml columns prepared by G-75 Sephadex (Sigma Chemical, Co.). Low interspecies cross-reactivity of the GH and PRL assays is shown in Fig. 5 columns prepared by G-75 Sephadex (Sigma Chemical, Co.). Low inter
differences between strains or subtypes were evaluated using two-tailed Student’s t test (nonnormally distributed variables). Tumor volumes were transformed by cube roots for the purpose of linearization. Linear growth models (intercept and slope) were estimated for changes in tumor volume and weight across time, using maximum likelihood methods and a mixed models approach similar to repeated measures ANOVA. The covariance structure was estimated with an autoregressive model. Analyses were performed using SAS version 9.1. For all tests, statistical significance was set at P < 0.05.

Results

EGFR expression and function in pituitary tumor cell lines. Immunoprecipitation with a specific EGFR antibody (ab2430) and subsequent immunoblotting (1005) revealed the expression of the 170 kDa EGFR in GH3 cells, whereas expression was not detected in MMQ and AtT20 pituitary cells (Fig. 1A). Similar to EGFR expression, immunoprecipitation and immunoblotting with Neu (C-18) antibody (sc-284), which detects ErbB2 and ErbB4, showed the expression ( ~ 185 kDa) of these related kinases in GH3 but not in MMQ or AtT20 cells (Fig. 1B). Mouse 3T3/A31 and human A431 cell lysates also showed the expression of these kinases.

In lacto-somatotroph GH3 cells, EGFR treatment (5 nmol/L) potently enhanced both baseline and serum-induced PRL mRNA abundance (Fig. 1C). EGFR induced PRL mRNA gene expression by ~ 4.1-fold (P < 0.05) at 48 hours. Serum alone also induced PRL expression ~ 3.2-fold, and cotreatment with serum and added EGF
further enhanced this effect to >6-fold (Fig. 1C). EGF modestly attenuated GH mRNA expression (Fig. 1C), and the growth factor did not induce S phase entry (Supplemental Fig. S1B). In contrast to the observed effects in GH3 cells, EGF treatment for up to 48 hours did not alter AtT20 cell POMC gene expression (data not shown).

Effect of gefitinib on pituitary tumor cell proliferation and gene expression. Treatment with increasing concentrations of gefitinib (0.1–10 μmol/L) dose-dependently attenuated serum-induced S phase entry in GH3 cells (Fig. 2B) and suppressed serum-induced Ptg1 mRNA expression (Fig. 2A). Gefitinib treatment modestly stimulated baseline and serum-induced GH, and inhibited PRL mRNA expression (Fig. 2A).

In contrast to GH3 cells, the percentage of AtT20 cells in S phase as well as serum-induced Ptg1 mRNA expression (>2.5-fold) was unaffected by increasing concentrations of gefitinib (0.1–10 μmol/L; data not shown). Gefitinib also did not alter POMC mRNA expression at 24 and 48 hours in AtT20 cells (data not shown).

The dose-dependent effects of gefitinib on GH3 and AtT20 cell proliferation are shown in Fig. 3C. Cells were incubated for 4 days with increasing doses of gefitinib. Doses as low as 0.1 μmol/L led to a ~15% (P < 0.001) attenuation of GH3 cell number, with a dose-dependent decrease of ~72% (P < 0.001) at 10 μmol/L. AtT20 cell numbers were not altered at these doses.

The observed EGF-induced lactotroph phenotype in GH3 cells (Fig. 1C) was reversed by pretreating cells with gefitinib prior to induction with EGF (Fig. 2D). The dose-dependent effects of gefitinib on the EGF-mediated lacto-somatotroph phenotype were evident at concentrations (5–10 μmol/L) which induced GH mRNA levels, whereas the lowest concentration tested (0.1 μmol/L) selectively abrogated EGF-induced PRL gene expression.

ErbB family member expression in GH3 cells. Induction of GH3 cells with EGF (5 nmol/L; Fig. 3Aa) or serum (data not shown) rapidly (within 5 minutes) induced tyrosine phosphorylation of several proteins sized >160, ~95, ~70, and ~40 kDa. As shown in Fig. 3A, proteins sized ~170 to 180 kDa were most prominently phosphorylated, corresponding to the approximate size of EGFR and related receptor members. EGF, but not serum, also induced tyrosine phosphorylation of proteins ~55 kDa in size, whereas phosphorylation by serum was more sustained (>2 hours; data not shown), as compared with the transient EGF effects (5–15 minutes). The dose-dependency of gefitinib on EGF-induced tyrosine phosphorylation is shown in Fig. 3Ab.

Activation of ErbB receptor members was confirmed by immunoprecipitation with receptor-specific antibodies (Fig. 3B). EGF (5 nmol/L) induced tyrosine phosphorylation of EGFR which was blocked by gefitinib pretreatment (1 μmol/L; Fig. 3Ba). B104-1-1 murine fibroblasts transformed by constitutively active oncogenic rat neu variant p185<sup>neu</sup> were used as a positive control for immunoprecipitation with the monoclonal antibody 7.16.4 (Fig. 3Bb). In contrast to B104-1-1 cells, GH3 cell neu tyrosine phosphorylation was only observed in response to ligand activation, suggesting that the cells express the proto-oncogenic cellular homologue p185<sup>neu</sup> (Fig. 3Bb). Potent tyrosine phosphorylation of p185<sup>neu</sup> was observed both in response to EGF (an EGFR receptor ligand) and HRG (6 nmol/L, an ErbB3/4 ligand; Fig. 3Bd), indicating p185<sup>neu</sup> as the preferred ErbB family heterodimerization partner in this cell line. Gefitinib pretreatment

Figure 2. Dose-dependent effects of gefitinib on GH, PRL, and Ptg1 mRNA expression and cell proliferation. A to D, GH3 cells were serum-starved for 24 h and pretreated with gefitinib (Gf, 45 min) at indicated concentrations prior to induction. A, induction with serum (S, 15% horse serum, 2.5% FBS). Total RNA was extracted at 48 h and indicated target mRNA expression determined as for Fig. 1C. Representative of three independently performed experiments (bottom). B, 22 h after induction with serum, cells were fixed and cell cycle analysis performed by flow cytometry. Percentage of cells in G<sub>0</sub>-M phase (black columns), cells in S phase (white columns), and cells in G<sub>2</sub>-M phase of the cell cycle (gray columns). C, after overnight serum starvation, GH3 or AtT20 cells were pretreated with gefitinib and subsequently cultured in the presence of complete medium for 4 d. Cell counts were performed with a hemocytometer. D, induction with EGF (5 nmol/L). Total RNA was extracted at 48 h and indicated target mRNA expression determined as for Fig. 1C. * P < 0.05; ** P < 0.01. Representative of three independently performed experiments (bottom).
(1 μmol/L) abrogated EGF-induced p185<sup>c-neu</sup> activation (Fig. 3Bb). Potent ErbB3 tyrosine phosphorylation was detected in response to the receptor ligand HRG (Fig. 3Bd), whereas low levels of ErbB3 tyrosine phosphorylation were occasionally detected in response to EGF (shown in Fig. 3Bd), suggesting modest ligand-induced EGFR/ErbB3 heterodimer formation.

**GH3 cell proliferation signaling pathways.** To identify the signaling pathways involved in GH3 cell proliferation, we blocked key signaling molecules prior to serum-induced release into the cell cycle. Inhibitors were screened at concentrations of 10 μmol/L (data not shown). Blockade of Rho, Src, and JAK tyrosine kinases by Y-27632, PP2, or JAK inhibitor I did not alter serum-induced cell proliferation. However, inhibition of MEK, phosphoinositide-3-kinase (PI3K), and protein kinase C (PKC) with U0126, LY294002, and GF109203X suppressed the number of cells entering S phase.

To analyze the mechanisms for gefitinib-mediated inhibition of pituitary cell proliferation, the drug was tested together with specific pathway inhibitors (Fig. 3C). For these experiments, gefitinib was employed at doses of 7.5 μmol/L and the lowest active concentrations of U0126, LY294002 (5 μmol/L), and GF109203X (1 μmol/L) were used as derived from dose-response experiments (data not shown).

Gefitinib inhibited serum-induced cell proliferation (induction of G<sub>0-1</sub> phase versus serum alone) by ~1.2-fold (P < 0.001). U0126, GF109203X, or LY294002 also inhibited serum-induced cell proliferation by ~1.1-fold (P < 0.05), 1.2-fold (P < 0.01), and 1.1-fold

**Figure 3.** ErbB family expression and cell proliferation signaling pathways in GH3 cells. A and B, GH3 cells were serum-starved overnight prior to stimulation. Aa, EGF (5 nmol/L) time course; b, pretreatment with gefitinib (Gf, dose response) prior to induction with EGF (5 nmol/L) for 5 min; Western blot analysis was performed with a monoclonal pTyr (PY99) antibody. Subsequently, membranes were stripped and reblotted with α-actin antibody as a loading control. Arrows, approximate positions of ErbB family receptors. Ba–c, GH3 cells were pretreated with gefitinib (Gf, 1 μmol/L) for 45 min prior to induction with EGF (5 nmol/L) for 10 min. b, B104-1-1 cells were serum-starved overnight and treated with EGF (5 nmol/L) as indicated. c, serum-starved GH3 cells were treated with EGF (5 nmol/L) or HRG (6 nmol/L) for 10 min. Immunoprecipitations were performed with EGFR, p185 (Ab 7.16.4), and ErbB3 antibodies as indicated. Immunoblotting was performed with pTyr antibody (top). Subsequently, membranes were stripped with mild stripping buffer and reblotted with EGFR, Neu, and ErbB3 antibodies (bottom). C, GH3 cells were serum-starved for 24 h and pretreated with gefitinib (7.5 μmol/L) and/or U0126 (5 μmol/L), LY294002 (5 μmol/L), or GF109203X (1 μmol/L) for 30 min prior to induction with serum (15% HS, 2.5% FBS). Twenty-two hours after induction with serum, cells were fixed and cell cycle analysis performed by flow cytometry. The amount of serum-treated cells in G<sub>0-1</sub> phase (control) was set as 1. Relative levels of cells in G<sub>0-1</sub> phase were normalized to this control group. Columns, mean fold G<sub>0-1</sub> phase induction in the treatment groups of five independently performed experiments; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
(P < 0.001), respectively (Fig. 3C). Cotreatment with gefitinib and the aforementioned signaling blockers prior to serum induction led to further attenuation of cell proliferation. The results of combined EGFR and ERK or PI3K inhibition were within the expected additive range of single agent treatments, whereas greater-than-additive effects were observed for combined EGFR and PKC signaling blockade.

**Effects of gefitinib on EGF and serum-induced ERK and PDK1 signaling.** The time-dependent phosphorylation of EGFR and subsequent ERK1/2 activation are depicted in Fig. 4. Similar to results observed in the total phosphotyrosine blots (Fig. 3A), EGF-mediated ERK activation seemed to be transient, lasting for up to 60 minutes (Fig. 4A), whereas serum induced a more sustained effect (up to 4 hours; Fig. 4C), and also modestly stimulated PDK1 phosphorylation (peaking at 5 to 10 minutes; Fig. 4C). Pretreatment with gefitinib dose-dependently suppressed EGF-induced EGFR phosphorylation (Fig. 4B) and blocked both EGF and serum-induced ERK phosphorylation with a similar potency (Fig. 4B and D). EGF did not induce ERK phosphorylation in AtT20 cells (data not shown), consistent with no detectable EGFR expression (Fig. 1A) and gefitinib did not alter serum-induced PDK1 phosphorylation (Fig. 4D).

**Effects of EGFR blockade on lacto-somatotroph tumor growth and hormone expression.** To evaluate *in vivo* effects of gefitinib on lacto-somatotroph tumor growth and hormone regulation, GH3 cells were implanted subcutaneously into female athymic NCR-NU mice. In vehicle-treated animals, relative tumor volume increased progressively, achieving 26-fold change from baseline at 18 days. In gefitinib-treated animals, relative tumor volume was attenuated by ~50% overall (Fig. 5Aa). Gefitinib treatment reduced absolute tumor volume from 865 ± 94 mm³ to 580 ± 110 mm³ versus controls by day 18 (Fig. 5Ab). Tumor volume increase was partially attributable to the emergence of an infiltrating inflammatory cell response by day 18 in gefitinib-treated animals (Fig. 5Ab).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Effects of EGF or serum and gefitinib on EGFR, ERK, and PDK1 activation. GH3 cells were serum-starved overnight and treated with EGF (5 nmol/L; A) or serum (15% horse serum, 2.5% FBS; C) for the indicated times or pretreated with gefitinib (Gf; indicated concentrations) prior to induction with EGF (B) or serum (D) for 10 min. Immunoprecipitation (IP) was performed with an EGFR antibody (ab2430) and immunoblotting was performed with pTyr antibody (first panels, A and B). Subsequently, membranes were stripped and reblotted with EGFR (sc-03) antibody (second panels, A and B). ERK1/2 phosphorylation, total ERK1/2 load, PDK1 phosphorylation, and total β-actin load as detected by Western blot in total protein extracts (A–D). Representative of three independently performed experiments.
correlated with postmortem tumor weight at day 18 (0.88, \( P < 0.001 \)). Analysis of all tumors \((n = 31)\) showed estimated slope values of 0.33 and 0.25, respectively, i.e., slower growth rate in the drug-treated group \((P < 0.05)\).

Vehicle-treated animals harboring tumors gained weight \((\sim 18\%)\) as compared with age-matched mice not injected with tumor cells (slope for mean weight increase per day, 0.27; Fig. 5la). Gefitinib treatment slowed the excessive weight gain, starting at day 8, and peaking at the end of the experiment (slope value, 0.04; \( P < 0.001 \)). Differences in body weight \((>4\) g) could not be ascribed to differences in tumor weight \((\sim 50\) mg). Although anorexic effects of gefitinib cannot be excluded, at maximum tolerated dose \((150\) mg/kg) in NCR-NU mice, weight loss does not exceed 10% \((22, 23)\), and weight loss was not observed compared with vehicle-treated animals not harboring tumors. Gefitinib treatment decreased serum GH, PRL, and IGF-I levels to 74% (not significant), 75% \((P = 0.036)\), and 66% \((P = 0.011)\), respectively (Fig. 5Ba).

Immunohistochemical analysis confirmed both EGFR and PRL expression in tumor tissue (Figs. 5C and 6B). EGFR immunoreactivity was especially prominent in peripheral areas of the tumor (Fig. 5Ca and b). Heterogeneous EGFR staining was also observed in tumor cells within the solid core area (Fig. 5Ca and c), whereas
central necrotic tissue was not immunoreactive for EGFR (Fig. 5Cd), serving as an internal negative control.

Tumor protein and RNA samples were paired in vehicle and gefitinib-treated animals according to tumor volumes. Western blot of tumor protein samples revealed ~23% (P < 0.05) decreased tumor ERK1/2 phosphorylation from gefitinib-treated animals (n = 16; Fig. 6Ad and b). Q-PCR also showed decreased PRL mRNA levels in four of six tumors, and decreased Ptgt1 mRNA expression in five of six volume-matched paired tumor samples. Overall, intratumoral PRL and Ptgt1 gene expression decreased by 35% (not significant) and 37% (P < 0.05), respectively (Fig. 6Ac). These results were confirmed by Northern blots of paired tumor extracts with sufficient RNA available (Fig. 6Ad). Intratumoral GH and VEGF-A mRNA levels assessed by Q-PCR (Fig. 6Ac) were not altered by treatment. In contrast, PRL immunoreactivity was suppressed in tumor cells after drug treatment (Fig. 6B).

**Discussion**

EGFR expression has been detected in normal anterior pituitary hormone-secreting cells (24), and EGF induces cell proliferation of nontumorous pituitary cells (25, 26). EGF induces lactotroph differentiation (27, 28), and targeted expression of a dominant negative EGFR in transgenic mice blocks both somatotroph and...
lactotroph development (29). Pituitary TGFs, an EGFR ligand, is up-regulated prior to the development of estrogen-mediated lactotroph hyperplasia in rats (30), and lactotroph TGFs overexpression in transgenic mice results in hyperplasia and adenoma formation (31), suggesting a role for EGFR in lactotroph tumorigenesis. Varying levels of EGFR binding have been reported in human pituitary tumors (10, 32–34), particularly in invasive adenomas (34) or carcinomas (13).

Immunoprecipitation and Western blotting confirmed that EGFR protein is expressed in lacto-somatotroph GH3 cells (35). As neither MMQ or AtT20 corticotroph cells express EGFR family members (Fig. 1A and B), the nonresponsiveness of these cell lines to the tested ligands and drugs was not surprising. In contrast, our results confirmed EGF-mediated induction of the lactotroph phenotype in GH3 cells, with marked induction of PRL transcription. The specificity of EGFR signaling was shown by suppression of EGF-mediated PRL induction by gefitinib (as low as 0.1 μmol/L). EGFR inhibition with gefitinib also reversed serum-induced lactotroph differentiation and cell proliferation, indicating a role for EGFR signaling in the mitogenic response. These results suggested that additional serum factors may lead to EGFR activation, which in turn, is required for PRL gene transcription and induction of cell proliferation.

ErbB (HER) family receptor overexpression, ligand binding, or oncogenic mutations lead to homodimerization or heterodimerization and subsequent induction of kinase activity. Several EGFR, ErbB3, and ErbB4 ligands have been identified, and ErbB2 is the preferred dimerization partner. The specific pattern of dimer formation determines the specificity of downstream signaling (for review see ref. 9). Monoclonal antibodies directed against extracellular domains of ErbB receptors as well as small molecule tyrosine kinase inhibitors allow targeted inhibition of tumor ErbB receptor signaling (9).

As ErbB receptors are expressed in rat GH3 cells (Fig. 1A and B), we examined mechanisms for ErbB family expression and activation. The rat neu oncogene encodes p185<sub>neu</sub> with intrinsic tyrosine kinase activity, which differs from its cellular homologue p185<sup>−<sub>neo</sub></sup> by a single transmembrane amino acid substitution (Val<sup>664 → Glu</sup>), resulting in constitutive activity of the intrinsic kinase (36). With the use of the monoclonal antibody 7.16.4, which reacts specifically with rat p185 molecules, we observed tyrosine phosphorylation of the precipitated receptor only after ligand induction, suggesting the expression of proto-oncogenic cellular p185<sup>−<sub>neo</sub></sup> in GH3 cells, compared with oncogenic and constitutively active p185<sup>neo</sup> expression in B104-1-1 controls (Fig. 3B). Gefitinib prevented EGF-induced tyrosine phosphorylation of EGFR and p185<sup>−<sub>neo</sub></sup>. In addition to the activation of ErbB3, induction with the ErbB3 ligand heregulin also activated p185<sup>−<sub>neo</sub></sup>, indicating that p185<sup>−<sub>neo</sub></sup> was the preferred dimerization partner in GH3 cells. However, the occasional detection of low-level ErbB3 tyrosine phosphorylation in response to EGF (an EGFR ligand) also suggests the potential of EGFR/ErbB3 heterodimer formation in GH3 cells.

EGFR signaling involves diverse cell-specific intracellular pathways and patterns of dimer formation (9), and we show that EGFR-related pathways activated by serum-induced GH3 cell proliferation include ERK, PI3K, and PKC. Combined treatment with gefitinib and the PKC inhibitor GF109203X resulted in marked induction of the G<sub>0</sub><sub>1</sub> phase (Fig. 3C), suggesting that the antiproliferative effects of EGFR and PKC inhibition are mediated by blockade of different signaling molecules. However, given the heterogeneity of PKC signaling (37), and the broad PKC isoform blockade achieved, specific involvement of particular PKC isoforms or novel isoform members cannot be excluded, as described for EGF-mediated regulation of the PRL promoter in GH4/GH4C1 cells (38).

As the results of combined EGFR and ERK or PI3K inhibition were within the expected additive range of monotherapies (Fig. 3C), we examined the direct effects of EGF and serum on EGFR/ERK and PKD1 (which mediates PI3K-dependent Akt phosphorylation; ref. 39) activation. Both EGF and serum induced rapid ERK phosphorylation, whereas serum activated PKD1, with sustained ERK activation, similar to patterns observed for tyrosine phosphorylation. Thus, transient EGFR-mediated ERK activation may be sufficient for the induction of PRL gene expression; however, sustained ERK phosphorylation and associated nuclear accumulation (40–42) as well as stimulation of other signaling pathways such as PI3K are necessary for serum induction of pituitary cell mitogenesis. The duration and strength of ERK activation elicits different cellular growth responses, i.e., proliferation versus differentiation (43). Concentrations of gefitinib as low as 0.1 μmol/l effectively suppressed EGF and ERK phosphorylation, but not PKD1. Thus, the results shown here suggest that the blockade of EGFR/ERK, and not PI3K signaling, mediate gefitinib inhibition of GH3 cell proliferation.

Injection of nude mice with GH3 cells resulted in the formation of large tumors and increased circulating serum PRL, GH, and IGF-I levels. Vehicle-treated animals harboring tumors showed increased weight gain during the course of the experiment, likely due to excessive GH and IGF-I levels. Treatment with gefitinib decreased tumor volume, serum hormone levels, and an attenuated weight curve comparable to that of non–tumor-bearing controls. In vivo antiproliferative activity of gefitinib was evidenced by lowered slope values for mean tumor volume increase in treated animals, and reduced Ptg mRNA expression in ex vivo tumor tissue. The failure of EGFR inhibition to completely block the growth of already established tumors can be ascribed to dose-related tumor drug penetration, heterogeneity of EGFR expression, and possible receptor down-regulation in central tumor areas with high cell density, as shown in Fig. 5C. Similar to the in vitro results, ex vivo analysis showed down-regulation of PRL but not GH mRNA expression. Thus, reduced peripheral tumor GH/IGF-I levels were likely the result of tumor load reduction, whereas serum PRL reduction likely occurred consequent to intratumor PRL gene and protein suppression. In vivo involvement of EGFR/ERK signaling was confirmed by demonstrating the reduction of ERK activity in treated tumor samples.

The results shown here suggest that EGFR inhibition could be useful for treating a subset of aggressive pituitary tumors with established EGFR expression, even if expression levels are relatively low, as EGFR expression levels do not necessarily predict gefitinib sensitivity (22, 44, 45). The results highlight the potential for screening postoperative pituitary tumor specimens for ErbB family expression. Inhibition of EGFR homodimerization and/or heterodimerization may block direct mitogenic signals by ErbB ligands or inhibit ErbB receptor transactivation by other stimuli (46, 47) required for induction of cell proliferation. In addition to the beneficial effects of tumor load reduction, potent inhibition of EGFR-mediated PRL gene expression could abrogate dramatic PRL elevations seen in patients with malignant prolactinomas. These infrequent neoplasms usually exhibit a slowly progressive malignant phenotype with metastasis, whereas resisting most forms of conventional therapy including dopamine agonists,
surgery, and radiation (7, 8). In addition to recent reports using the alkylating compound temozolomide (48, 49), or new generation PRL receptor antagonists (50), targeted EGFR inhibition could represent an alternative treatment for patients with EGFR-expressing malignant prolactinomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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