Cross-talk between G Protein–Coupled Receptor and Epidermal Growth Factor Receptor Signaling Pathways Contributes to Growth and Invasion of Head and Neck Squamous Cell Carcinoma

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Abstract

G protein–coupled receptors (GPCR) and the epidermal growth factor receptor (EGFR) are often both overexpressed and contribute to the growth of cancers by activating autocrine pathways. GPCR ligands have been reported to trigger EGFR signaling via receptor cross-talk in cancer cells. Here, we show that GPCR ligands prostaglandin E2 (PGE2) and bradykinin (BK) activate EGFR signaling. Inhibition of EGFR using several strategies, including small-molecule inhibitors and an EGFR-specific antibody, resulted in partial attenuation of signaling downstream of EGFR. PGE2 and BK triggered EGFR signaling by increasing selective autocrine release of transforming growth factor-α (TGF-α). Inhibition of tumor necrosis factor-α–converting enzyme abrogated BK- or PGE2-mediated activation of EGFR signaling. Both PGE2 and BK stimulated head and neck squamous cell carcinoma (HNSCC) invasion via EGFR. Treatment of HNSCC cells with the BK antagonist CU201 resulted in growth inhibition. The combination of CU201 with the EGFR small-molecule inhibitor erlotinib resulted in additive inhibitory effects on HNSCC cell growth in vitro. Inhibition of the PGE2 synthesis pathway with sulindac induced HNSCC cytotoxicity at high doses (EC50, 620 μmol/L). However, combined inhibition of both EGFR with the tyrosine kinase inhibitor erlotinib and GPCR with sulindac at low doses of 6 and 310 μmol/L, respectively, resulted in synergistic killing of HNSCC tumor cells. Combined blockade of both EGFR and GPCRs may be a rational strategy to treat cancers, including HNSCC that shows cross-talk between GPCR and EGFR signaling pathways. (Cancer Res 2006; 66(24): 11831-9)

Introduction

The epidermal growth factor (EGF) receptor (EGFR) is up-regulated in head and neck squamous cell carcinomas (HNSCC) where increased expression is associated with reduced survival (1). We previously showed that targeting EGFR decreases HNSCC proliferation and invasion (2). EGFR is emerging as an important therapeutic target for several epithelial tumors, including HNSCC. Results of clinical trials to date suggest that antitumor effects are primarily observed when EGFR blockade is combined with standard cytotoxic therapies, such as radiation (3). The modest benefit of EGFR blockade when monoclonal antibodies or tyrosine kinase inhibitors are administered as single agents could be attributed to compensation by other signaling pathways that are independent of EGFR.

G protein–coupled receptors (GPCR) are ubiquitously expressed in epithelial cells. Several GPCR ligands have been shown to activate the EGFR pathway contributing to HNSCC carcinogenesis. We previously reported that stimulation of the gastrin-releasing peptide (GRP) receptor activates EGFR and modulates HNSCC growth and invasion (4). In addition, EGFR activation in response to GPCR ligands, including lysophosphatidic acid (LPA) and thrombin, has also been reported in HNSCC cells (5). We and others have previously shown that GPCR ligand stimulation results in increased autocrine secretion of EGFR ligands, including transforming growth factor-α (TGF-α) and amphiregulin (AR; refs. 4, 6). GPCR ligands prostaglandin E2 (PGE2) and bradykinin (BK) are inflammatory molecules that have been previously reported to contribute to the proliferation of colon and breast cancer cells, respectively (7, 8). PGE2 has been reported to activate EGFR in tumors, including colon cancer, by activating other molecules, including Src and matrix metalloproteinases (MMP; refs. 6, 9). BK also has been reported to stimulate proliferation in breast cancer cells via EGFR (10). Combined inhibition of EGFR and GPCR may lead to enhanced antitumor effects compared with single agents targeting EGFR alone.

Several studies have shown that decreased PGE2 synthesis using cyclooxygenase (COX)-2 inhibitors results in growth inhibition of cancers, including HNSCC (11, 12). Inhibition of BK with the BK receptor antagonist CU201 has been previously reported to inhibit the growth of lung cancer cells (13). The effects of BK and PGE2 on EGFR signaling and the antitumor efficacy of combined EGFR and BK receptor inhibition have not been investigated in HNSCC cells. The goal of the present study was to determine the role of the GPCR ligands PGE2 and BK in mediating HNSCC growth and invasion via EGFR-dependent and EGFR-independent pathways. The effects of PGE2 and BK stimulation on EGFR and downstream signaling, growth, and invasion were investigated. Finally, we examined the antitumor efficacy of combined targeting of EGFR and GPCR as a potential treatment regimen for HNSCC.
Materials and Methods

Cell lines. HNSCC cell lines 1483 and PCI-37A of human origin were maintained in DMEM plus 10% fetal bovine serum (FBS) at 37°C with 5% CO2 as described previously (14, 15). Primary cultures from murine embryonic fibroblasts derived from EGFR knockout mice (KO) mice or wild-type (WT) littermates (The Jackson Laboratory, Bar Harbor, ME) at 16.5 embryonic days were maintained in DMEM plus 10% FBS at 37°C with 5% CO2 as described previously (4).

Reagents. EGFR-specific tyrosine kinase inhibitors AG1478 and AG9 were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Erlotinib and gefitinib were obtained from OSI Pharmaceuticals (Melville, NY) and AstraZeneca Pharmaceuticals (Wilmington, DE), respectively. Anti-EGFR antibody C225 was obtained from Imclone Systems, Inc. (New York, NY). The small-molecule c-Src inhibitor PD0180970 was obtained from Pfizer, Inc. (New York, NY). Human PGE2 and BK were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Recombinant human EGF was obtained from Oncogene Research Products (Boston, MA). The antibody used to coimmunoprecipitate EGFR was obtained from Upstate Biotechnology (Lake Placid, NY). For immunoblotting, anti-EGFR antibody from Transduction Laboratories (Lexington, KY) was used. Phosphorylation of EGFR was detected by coimmunoprecipitation for EGFR followed by immunoblotting using phosphotyrosine antibody PY99 from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p44/42 mitogen-activated protein kinase (MAPK) and phosphorylated p44/42 MAPK were from Cell Signaling Technology, Inc. (Danvers, MA). Neutralizing antibodies to TGF-α and heparin-binding EGF (HB-EGF) were obtained from Oncogene Research Products (Lafayette, CO). The antibody used to coimmunoprecipitate EGFR was obtained from Upstate Biotechnology followed by immunoblotting for phosphotyrosine antibody PY99 from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p44/42 mitogen-activated protein kinase (MAPK) and phosphorylated p44/42 MAPK were from Cell Signaling Technology, Inc. (Danvers, MA). Neutralizing antibodies to TGF-α and heparin-binding EGF (HB-EGF) were obtained from Oncogene Research Products. Neutralizing antibody to AR was obtained from R&D Systems (Minneapolis, MN). HEMA3 cell staining kit was purchased from Fisher Scientific (Hampton, NH). The BK antagonist C221 was synthesized as reported previously (13). Sulindac was obtained from Sigma-Aldrich. Tumor necrosis factor-α–converting enzyme (TACE) small interfering RNA (siRNA) based on previously published sequences was obtained from Dharmacon International (Lafayette, CO; ref. 5). TGF-α ELISA kit was obtained from Oncogene Research Products.

Cell treatments. HNSCC cells and EGFR KO or EGFR WT fibroblasts (2 x 10^5/mL) were plated in 10 cm^2 plates. For mechanistic studies, cells were serum starved for 72 hours. During serum starvation, the medium was changed every 24 hours. For the experiments with inhibitors, cells were pretreated with AG9 or AG1478 (250 nmol/L), C225 (6 μg/mL), TGF-α (7 μg/mL), HB-EGF (50 μg/mL), AR (15 μg/mL), or PD0180970 (500 nmol/L) for 2 hours. For TACE down-modulation, previously described TACE siRNA (104/mL) were plated in 10 cm^2 plates. For mechanistic studies, cells were serum starved for 72 hours. For the experiments with inhibitors, cells were pretreated with AG9 or AG1478 (250 nmol/L), C225 (6 μg/mL), TGF-α (7 μg/mL), HB-EGF (50 μg/mL), AR (15 μg/mL), or PD0180970 (500 nmol/L) for 2 hours. For TACE down-modulation, previously described TACE siRNA duplexes were used (5). Briefly, HNSCC cells (2 x 10^5) were transfected with TACE siRNA in the presence of LipofectAMINE 2000 (Invitrogen, Inc., Carlsbad, CA) and serum starved for 48 hours after transfection. Following treatments with inhibitors, HNSCC cells were stimulated with EGF (10 ng/mL), PGE2 (10 nmol/L), or BK (10 nmol/L) for 5 minutes to examine phosphorylation of EGFR and for 10 minutes to examine phosphorylation of MAPK. Cells were lysed as described previously (4). For in vitro proliferation studies, cells were serum starved for 24 hours following treatment with either PGE2 or BK (10 nmol/L) for 2 days. Cells were counted using vital dye exclusion.

In vitro invasion and cytotoxicity assays. In vitro invasion assays were carried out in growth factor–reduced Matrigel-coated Transwell chambers (BD Biosciences, San Jose, CA). Briefly, HNSCC cells 1483 (5,000 per well) were plated in the Transwell chamber in the presence of 2.5% serum, 10 nmol/L PGE2, or 10 nmol/L BK in the presence or absence of erlotinib (6 μmol/L). Cells were incubated at 37°C with 5% CO2 for 24 hours. Cells that invaded the Transwell chamber were fixed and stained using the HEMA3 staining solutions according to the manufacturers’ instructions. The number of cells that invaded the Matrigel was determined under ×200 magnification.

For in vitro cytotoxicity assays, 1483 cells were treated 24 hours after plating with EGF5 tyrosine kinase inhibitor gefitinib (3 μmol/L) and/or BK antagonist C221 (4 μmol/L) or a combination of erlotinib (6 μmol/L) and/or sulindac (310 μmol/L) at varying concentrations for 72 hours followed by cell counting determinations using vital dye exclusion.

Immunoblotting. Cell lysates were clarified by centrifugation at 14,000 rpm for 5 minutes followed by protein estimation. Protein (30 μg) was resolved on an 8% SDS-PAGE gel and immunoblotted for phosphorylated MAPK followed by MAPK (Cell Signaling Technology, Beverly, MA). Immunoprecipitation for EGFR was carried out with anti-EGFR antibody obtained from Upstate Biotechnology followed by immunoblotting for phosphotyrosine (PY99) and total EGFR (Transduction Laboratories). The same membrane was stripped and probed for EGFR to show equal loading (Transduction Laboratories). Autoradiographs were scanned and subjected to densitometry analyses using DigiDoc 1000 digital imaging system (Alpha Innotech, San Leandro, CA). For the phosphorylated and total p42/44 MAPK blots, the cumulative intensities of both the p42 as well as the p44 bands were estimated.

Statistical analysis. Statistical determinations were done using the StatXact (version 6) software (Cytel Software Corp., Cambridge, MA). For two-way comparisons for independent samples, the exact Wilcoxon test was used to compare two groups. For comparison of multiple independent samples, the Kruskal-Wallis test for equality of the three groups was used.

Results

PGE2 and BK activate EGFR in HNSCC cells. PGE2 and BK are inflammatory molecules that have been previously reported to contribute to proliferation in breast and colon carcinoma (6, 10). The concentration of PGE2 and BK used in these studies was determined by treating HNSCC cells with increasing doses of the ligands. The minimum dose at which both ligands stimulated MAPK activation in HNSCC cells was 10 nmol/L. To determine the effects of PGE2 and BK on EGFR activation, HNSCC cells (1483) were stimulated with either PGE2 or BK for 5 minutes. Cell lysates were examined for phosphorylated EGFR and MAPK by immunoblotting. Similar to our previous findings with GRP, a 1.5- to 2-fold induction of EGFR phosphorylation was observed on ligand stimulation with either GPCR ligand (P = 0.05; Fig. 1A). Both PGE2 and BK also activated MAPK (3- to 4-fold increase over the baseline) in HNSCC cell lines (1483 and PCI-37A; Fig. 1B). These data show that PGE2 or BK stimulation activates the EGFR signaling axis in HNSCC.

PGE2 and BK activate MAPK via both EGFR-dependent and EGFR-independent pathways. We previously showed that GRP-mediated MAPK activation occurs primarily downstream of EGFR in HNSCC (4). However, EGFR-independent activation of MAPK by BK has been reported in lung cancer cells (16). To examine the role of EGFR in PGE2- or BK-mediated activation of MAPK, we pretreated serum-starved HNSCC cells (1483) with the EGFR-specific small-molecule inhibitor AG1478 (200 nmol/L) or the inactive isomer AG9 (200 nmol/L). Cells were treated with 10 nmol/L of either PGE2 or BK in the presence or absence of the EGFR inhibitor. Cell lysates were examined for MAPK phosphorylation by immunoblotting. Both PGE2 and BK stimulated MAPK phosphorylation in the presence of EGFR inhibitors, albeit to a lesser degree, indicating both EGFR-dependent and EGFR-independent mechanisms of MAPK activation in HNSCC cells by PGE2 or BK (P > 0.05; Fig. 2A). It is possible that the p42 MAPK band is regulated to a greater extent compared with the p44 MAPK band. There was a 2.5-fold reduction in the level of p42/44 MAPK phosphorylation by PGE2 in the presence of the EGFR inhibitor compared with the control (AG9) compound. Stimulation with BK generated a 2.5-fold activation of p42/44 MAPK in the presence of EGFR inhibitor compared with 4-fold activation in the presence of the control compound (AG9). These data show that PGE2 and BK may activate MAPK via both EGFR-dependent and EGFR-independent pathways in HNSCC cells.

To verify the requirement of EGFR for GPCR ligand-induced MAPK phosphorylation in a genetically defined system, the above
EGFR, triggering signaling cascades downstream of EGFR, including MAPK (4). To elucidate the mechanism whereby GPCR ligands PGE2 and BK activate EGFR, HNSCC cells were pretreated with an EGFR-specific antibody (C225) that binds the extracellular ligand-binding domain of EGFR, preventing ligand binding. Cells were subsequently stimulated with either PGE2 or BK (10 nmol/L for 10 minutes). Although GPCR-mediated MAPK phosphorylation was partially abrogated on EGFR extracellular domain inhibition, the attenuation in phosphorylation was not significant (P = 0.42; Fig. 2C). This indicates that MAPK may be phosphorylated by EGFR-dependent as well as EGFR-independent mechanisms in HNSCC.

**TGF-α, but not HB-EGF or AR, is released by PGE2 or BK stimulation.** We and others have previously shown that GPCR ligand stimulation of HNSCC cells facilitates cleavage of membrane-bound EGFR proligands TGF-α, AR, and/or HB-EGF depending on the specific GPCR ligand used (4, 18). Neutralizing antibodies to TGF-α, but not HB-EGF or AR, blocked PGE2- and BK-induced MAPK phosphorylation in HNSCC cells (P = 0.05 on comparing PGE2- or BK-stimulated levels of MAPK phosphorylation with those in the presence of TGF-α neutralizing antibody; Fig. 3A–C). Release of mature ligand increases phosphorylation of EGFR, triggering signaling events downstream of EGFR. These results suggest that, in HNSCC cells, the specific EGFR ligand activated depends on the precise GPCR stimulus.

**Src family kinases and TACE are involved in PGE2- and BK-mediated signaling.** We previously reported that Src family kinases are involved in GRP-mediated activation of MMPs that cleave EGFR proligands from the membrane in HNSCC (19). To examine if Src family kinases contribute to PGE2- and/or BK-mediated MAPK activation, we treated HNSCC cells with a Src family kinase inhibitor PD0180970 for 2 hours before GPCR ligand stimulation. Inhibition of Src family kinases resulted in an attenuation of MAPK phosphorylation (P = 0.05; Fig. 4A).

We previously showed that GRP-mediated MAPK activation requires MMP activity (4). To determine the role of MMP in PGE2- and BK-stimulated activation of EGFR, we treated HNSCC cells with the broad-spectrum MMP inhibitor GM6001 and examined the consequences of GPCR stimulation on MAPK phosphorylation. In the presence of MMP inhibition, PGE2 or BK stimulation of HNSCC cells resulted in partial activation of MAPK. HNSCC cells (1483) were pretreated for 2 hours with the MMP inhibitor GM6001 (20 nmol/L) followed by stimulation with GPCR ligands PGE2 or BK. Both ligands only partially activated MAPK in the presence of GM6001 (data not shown). TACE has been previously reported to be involved in EGFR proligand cleavage (20, 21). To examine the role of TACE in PGE2- or BK-mediated EGFR activation, HNSCC cells 1483 were transfected with either TACE siRNA or a control green fluorescent protein (GFP) siRNA. It has been previously reported that TACE siRNA does not affect ADAM12 expression in HNSCC cells (5). In addition, we found that TACE siRNA did not alter ADAM10 mRNA expression levels (data not shown). After 48 hours of serum deprivation, cells were stimulated with either PGE2 or BK (10 nmol/L) for 10 minutes. Cell lysates were examined for MAPK phosphorylation and TACE expression by immunoblotting (Fig. 4B). In the presence of TACE down-modulation, MAPK activation was attenuated on PGE2 or BK stimulation. In addition, we analyzed the supernatants from cells transfected with TACE or GFP (control) siRNA before BK stimulation for TGF-α levels by ELISA. TACE down-modulation reduced levels of TGF-α released into the supernatant on BK stimulation (P = 0.05; Fig. 4C). These results show that TACE contributes to the cleavage of TGF-α proligand on BK stimulation.

**Figure 1.** Phosphorylation of EGFR and MAPK by PGE2 and BK. Representative HNSCC cell lines were treated with EGF (10 ng/mL), PGE2 (10 nmol/L), or BK (10 nmol/L) for 10 minutes. A, cell lysates from HNSCC cell line 1483 were examined for phosphorylated EGFR (Phospho-EGFR) via immunoblotting. Cumulative results from three independent experiments show a significant increase in EGFR phosphorylation on PGE2 or BK stimulation compared with the vehicle control (P = 0.05). B, cell lysates from HNSCC cell lines 1483 and PCI-37A were examined by immunoblotting for phosphorylated p42/44 MAPK (p44/42 phospho MAPK). The experiment was carried out thrice for each cell line with similar results. Total EGFR and p42/44 MAPK levels show equal loading of protein.
PGE2 and BK stimulate HNSCC cell proliferation and invasion via EGFR. PGE2 and BK are inflammatory factors that have been previously reported to stimulate growth of colon or breast cancer cells, respectively (7, 10). The effect of PGE2 or BK on the growth of head and neck cancer cells has not been described previously. To determine the effects of PGE2 and BK on HNSCC proliferation, 1483 cells were stimulated with either 0.1 or 10 nmol/L of either PGE2 or BK for 3 days. Cell counts revealed that both ligands stimulated HNSCC cell proliferation compared with vehicle control–treated cells in a dose-dependent manner (P < 0.05; Fig. 5A).

To investigate if EGFR is required for PGE2 or BK to induce cell proliferation, EGFR KO and WT fibroblasts were serum starved for 72 hours before the addition of EGF (10 ng/mL), PGE2 (10 nmol/L), or BK (10 nmol/L). Cells were pretreated for 2 hours with AG1478 (200 nmol/L) or the control inhibitor AG9 (200 nmol/L). Partial attenuation of MAPK phosphorylation was not significantly different on EGFR inhibition in the presence of PGE2 or BK, indicating that there may be EGFR-independent phosphorylation of MAPK by both ligands (P > 0.05). Cumulative results from four independent experiments. B, cells derived from EGFR KO mice were treated with GPCR ligands (PGE2 or BK at 10 nmol/L) followed by immunoblotting for phosphorylated p42/44 MAPK and total p42/44 MAPK. Both PGE2 and BK activated MAPK in the absence of EGFR (P = 0.04). Cumulative results from three independent experiments. C, PGE2- or BK-stimulated MAPK activation is partially abrogated on inhibition of the EGFR extracellular ligand-binding domain. HNSCC cells 1483 were treated with C225 (6 μg/mL) for 2 hours followed by PGE2 or BK (both at 10 nmol/L) for 10 minutes and immunoblotted for phosphorylated p42/44 MAPK and total p42/44 MAPK. Inhibition of EGFR with C225 failed to completely abrogate PGE2- or BK-stimulated phosphorylation of MAPK, indicating that phosphorylation of MAPK may occur independent of EGFR (P = 0.42). Cumulative results from four independent experiments.
In addition to being mitogenic, both PGE2 and BK have been previously reported to stimulate invasion of cancer cells (9, 22). The role of PGE2 or BK in HNSCC invasion has not been reported. To determine whether PGE2 or BK stimulates invasion via EGFR, HNSCC cells were plated in Matrigel-coated invasion chambers with 10 nmol/L of PGE2 or BK. Additional wells with GPCR ligands were treated with the EGFR-specific inhibitor erlotinib (6 μmol/L). Cells were allowed to invade toward serum-containing medium, which served as a chemoattractant over 24 hours. Both PGE2 and BK induced invasion of HNSCC cells, an effect that was abrogated in the presence of EGFR inhibition (P < 0.05; Fig. 5C). This indicates that both PGE2 and BK mediate invasion via an EGFR-dependent mechanism.

**Combined inhibition of EGFR and BK or PGE2 improves growth inhibition in HNSCC.** It has been suggested that targeting the EGFR axis at multiple levels may improve the antitumor effects of molecular targeted therapies (23). Our findings suggest that PGE2 and BK modulate HNSCC invasion primarily via EGFR. We postulated that combined targeting of both receptors may augment the antitumor effects of EGFR blockade. HNSCC cells were treated with increasing concentrations of the BK antagonist CU201 (0–16 μmol/L) for 72 hours. The EC50 obtained on CU201 treatment was found to be 8 μmol/L (Fig. 6A). This dose is comparable with that reported previously in lung cancer cells (13). Next, HNSCC cells were treated with gefitinib (3 μmol/L) in combination with CU201 (4 μmol/L) for 72 hours. Additive growth-inhibiting effects were observed on combined inhibition of both receptors (Fig. 6B). Similar results were obtained on combining CU201 with erlotinib, another EGFR-specific small-molecule inhibitor (data not shown). Because PGE2 also had a growth-stimulatory effect, we examined the efficacy of combined inhibition of PGE2 and EGFR in HNSCC using the COX inhibitor sulindac. We elected to use the nonselective COX inhibitor sulindac rather than a COX-2 inhibitor, such as celecoxib, due to the recent cardiac toxicities of COX-2 inhibition and, therefore, the limited application for clinical development (24). HNSCC cells (1483) were exposed to increasing doses of sulindac for 72 hours. HNSCC cells were sensitive to sulindac treatment with a EC50 dose of 620 μmol/L (Fig. 6C). Combining sulindac (310 μmol/L) and EGFR inhibitor erlotinib (6 μmol/L) for 72 hours resulted in synergistic growth inhibition in HNSCC cells (Fig. 6D).

**Discussion**

EGFR is expressed at high levels in HNSCC where signaling through this receptor contributes to cell survival, proliferation, and invasion. Inhibition of EGFR alone, although highly promising in preclinical models, has resulted in limited antitumor effects...
(response rates <10%) when tested as a monotherapy in clinical trials (25). In addition to receptor tyrosine kinases, GPCRs regulate the responsiveness of cancer cells to external stimuli. It has become increasingly evident that complex interactions between EGFR and GPCRs generate signaling networks that confer an aggressive tumorigenic phenotype in cancer cells (26, 27). GPCR-mediated EGFR activation has been shown in breast and prostate cancer cell lines (28, 29). Several GPCR ligands, including LPA, carbachol, thrombin, and GRP, induce HNSCC proliferation and motility via transactivation of EGFR (5, 18).

Proposed intracellular mechanisms of EGFR transactivation by GPCRs have included roles for the serine/threonine kinase PKC (32), the nonreceptor tyrosine kinases of the Src family (27), and increased intracellular calcium levels (33). The mechanism of activation of EGFR by GPCRs seems to depend on the cell type and the specific GPCR ligand involved. Originally thought to be exclusively regulated by intracellular pathways, our results corroborate evidence suggesting that the EGFR extracellular domain is involved in GPCR-EGFR cross-talk (28). Several EGFR ligands have been detected in HNSCC cells, including betacellulin and AR, in addition to EGF, TGF-α, and HB-EGF (34). We have previously shown that down-modulation of TGF-α results in inhibition of HNSCC growth, indicating that TGF-α is primarily involved in autocrine activation of EGFR in head and neck cells (35). Further, enhanced TGF-α levels in HNSCC tumors correlate with...
decreased survival (36). Emerging evidence suggests that, in addition to TGF-α, HB-EGF and/or AR are involved in GPCR ligand-mediated signaling. Activation of EGFR by GRP in lung cancer cells occurs via TGF-α and HB-EGF, whereas in HNSCC cells TGF-α and AR are involved (4, 37). Our findings suggest that only TGF-α, and not HB-EGF or AR, is involved in EGFR activation by PGE2 or BK. Thus, depending on the type of GPCR ligand involved, autocrine release of TGF-α either alone or along with other EGFR ligands, including HB-EGF or AR, may be increased. Autocrine release of EGFR ligands results from MMP activation in cells.

Membrane-bound EGFR proligands are processed and cleaved by metalloproteinases into functional ligands that bind to the receptor. Depending on the type of GPCR under investigation, EGFR proligands were reported to be activated by ADAM10, ADAM15, or ADAM17 in bladder and kidney cancer cells (38). We and others have previously shown that the TACE or ADAM17 is involved in GPCR-mediated activation of transmembrane EGFR ligand precursors pro-TGF-α, pro-AR, and pro-HB-EGF (5, 19, 21, 38). Our current results corroborate these studies in that down-modulation of TACE by siRNA in HNSCC cells attenuated PGE2- and BK-mediated signaling events downstream of EGFR. In addition to TACE, our data show that Src family kinases are involved in PGE2- and BK-mediated signaling in HNSCC. This result is consistent with our previous finding that Src family kinases are involved in GRP ligand-mediated activation of EGFR in HNSCC (19). These cumulative findings implicate Src family kinases as potential therapeutic target in HNSCC. Several Src tyrosine kinase inhibitors are under clinical development, including AZD0530, SKI-606, and BMS-354825 (39).

It is well established that activation of EGFR and downstream signaling molecules, including MAPK, results in increased proliferation and invasion of HNSCC cells (4, 40). PGE2 and BK have been implicated in the growth of colon and breast carcinoma, respectively (7, 8). Here, we have shown that GPCR ligands PGE2 and BK induce mitogenic effects in HNSCC cells. To determine the role of EGFR activation in GPCR-induced mitogenesis, we treated cells derived from EGFR KO mice with PGE2, BK, or EGF. Treatment of cells derived from WT littermates with either ligand modestly stimulated growth. However, EGF, PGE2, and BK failed to stimulate growth of EGFR KO cells. In addition, treatment of EGFR KO or WT cells with serum stimulated growth, showing that these cells can be stimulated to proliferate in vitro. These results suggest that EGFR tyrosine kinase activity primarily mediates the growth-stimulatory effects of GPCR in murine embryonic fibroblasts. Although this finding cannot be directly extrapolated to human HNSCC cells, it serves as a proof of principle that EGFR may mediate the proliferative effects of GPCR ligands PGE2 and BK. Inhibition of EGFR in HNSCC cell lines over 72 hours resulted in cell death, so it was not possible to elucidate the EGFR-independent effects of GPCR ligands on HNSCC cell proliferation. In addition to mitogenesis, GPCRs have been implicated in invasion of cancer cells (38). PGE2 and BK have been reported to induce invasion in cholangiocarcinoma and in bladder cancer cells, respectively (22, 41). Both PGE2 and BK induced invasion of HNSCC cells through Matrigel-coated Transwell chambers. Inhibition of EGFR with low doses of small-molecule inhibitors erlotinib or gefitinib abrogated GPCR ligand-mediated invasion of HNSCC cells. These results show that GPCRs promote migration and invasion of HNSCC cells via activation of the EGFR signaling axis. Thus, PGE2 and BK may stimulate MAPK phosphorylation independent of EGFR, although combined activation of EGFR and GPCR pathways is required for maximal invasiveness of HNSCC. Thus, PGE2 and BK receptors represent potential targets for HNSCC intervention strategies.
Several peptide inhibitors are being developed to target GPCRs. CU201 is a BK antagonist that is currently being tested in clinical trials for lung cancer (13). We determined that HNSCC cells were sensitive to low doses of CU201 in vitro. This dose is comparable with previous reports of micromolar doses of CU201 being cytotoxic in lung cancer cells (42). Despite promising antitumor effects in preclinical models, EGFR inhibitors tested in clinical trials have shown limited antitumor effects when administered as a monotherapy (43). Development of resistance to EGFR inhibition via the activation of downstream signaling molecules via alternate receptor pathways may explain the limited antitumor efficacy (44). In this report, we have shown activation of MAPK by PGE2 and BK via EGFR-independent pathways. Thus, combined targeting of GPCRs and EGFR would be more effective in abrogating tumor growth in HNSCC cells. Indeed, combined treatment of HNSCC cells with a small-molecule EGFR inhibitor and the BK antagonist resulted in additive antitumor effects. Inhibition of the PGE2 synthesis pathway with COX-2 inhibitors has been reported to result in growth inhibition of lung cancer cells (45). Combined therapy with EGFR and COX-2 inhibitors resulted in antitumor effects in HNSCC cells (12). Here, we show that combined treatment of HNSCC cells with a small-molecule EGFR inhibitor and the nonselective COX inhibitor sulindac results in synergistic antitumor effects. Because GPCRs signal via both EGFR-dependent as well as EGFR-independent mechanisms, targeting both receptors simultaneously showed increased efficacy compared with inhibiting either receptor alone. These results support the development of therapeutic strategies that combine EGFR and GPCR targeting in HNSCC.

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Figure 6. Combined inhibition of EGFR and GPCR increases antitumor effects of single agents alone. A, dose-dependent growth inhibition of BK antagonist CU201 in HNSCC cells. HNSCC cells (1483) were treated with increasing doses of CU201 followed by cell counting 3 days later. B, increased growth inhibition with GPCR blockade combined with EGFR targeting. HNSCC cells were treated with gefitinib alone (3 μM/L) or in combination with CU201 (4 μM/L) followed by cell count determinations 72 hours later. C, dose-dependent growth inhibition with sulindac in HNSCC cells 1483. HNSCC cells were treated with increasing doses of sulindac followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) determinations after 72 hours. D, synergistic antitumor effects of combined inhibition of EGFR and PGE2 pathways. Treatment of HNSCC cells 1483 with erlotinib (6 μM/L) and sulindac (310 μM/L) resulted in synergistic antitumor effects after 72 hours. Percentage surviving cells were determined using the MTT assay.


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