EFFECTS OF PHARMACOLOGIC ANTAGONISTS OF EPIDERMAL GROWTH FACTOR RECEPTOR, PI3K AND MEK SIGNAL KINASES ON NF-κB AND AP-1 ACTIVATION AND IL-8 AND VEGF expression in human head and neck squamous cell carcinoma lines

Caren C. Bancroft, Zhong Chen, Jason Yeh, John B. Sunwoo, Ning T. Yeh, Sadhana Jackson, Chad Jackson and Carter Van Waes*

Tumor Biology Section, Head and Neck Surgery Branch, The National Institute on Deafness and Other Communication Disorders, The National Institutes of Health, Bethesda, MD, USA

We previously reported that expression of angiogenesis factors interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF) is promoted by coactivation of transcription factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) by interleukin-1α in human head and neck squamous cell carcinomas (HNSCC). However, expression of IL-1 receptor antagonist incompletely blocked reporter gene activity and cytokine expression, suggesting that other upstream signals may contribute to activation. Overexpression and activation of receptor-tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR), is detected in 90% of HNSCC, and EGFR inhibitors have been reported to inhibit IL-8 and VEGF expression, but the intermediary signal pathways and transcription factors by which EGFR modulates proangiogenic factors is unknown. EGFR can activate the phosphotidylinositol-3 kinase (PI3K) and mitogen-activated/extracellular signal-regulated kinase (MEK) pathways, which can potentially modulate activation of NF-κB and AP-1, respectively. In our study, we examined the effect of EGFR and antagonists of EGFR, PI3K and MEK on NF-κB and AP-1 activation and IL-8 and VEGF expression in HNSCC cell lines UM-SCC-9 and 11B in which EGFR is overexpressed and activated. Recombinant EGFR induced EGFR phosphorylation, activation of NF-κB and AP-1 reporter genes and IL-8 and VEGF expression, indicating that EGFR can mediate coactivation of both transcription factors and cytokine genes in HNSCC. EGFR antagonist PD153035 and anti-EGFR antibody C225 completely inhibited EGFR-induced reporter activity and cytokine expression, but only partially inhibited constitutive activity. MEK inhibitor U0126 preferentially blocked AP-1 activity and expression of both IL-8 and VEGF, while PI3K inhibitor LY-294002 or a dominant negative inhibitor-kB preferentially blocked NF-κB activation and expression of IL-8 but not VEGF. EGFR, PI3K and MEK antagonists inhibited growth of HNSCC. We conclude that antagonists of EGFR, PI3K and MEK signal pathways have inhibitory activity against EGFR-induced NF-κB and AP-1 activation, IL-8 and VEGF expression and growth by HNSCC. Published 2002 Wiley-Liss, Inc.

Key words: EGFR; NF-κB; AP-1; IL-8; VEGF; PI3K; MEK; HNSCC

Increased angiogenesis is critical to tumor progression and metastasis, and we and others have shown that expression of members of the C-X-C cytokine and vascular endothelial growth factor family such as IL-8, growth regulated oncogene-1 (Gro 1) and VEGF can promote angiogenesis, tumorigenesis and metastatic tumor progression.1-3 The expression of multiple factors with proangiogenic activity by cancer cells poses a significant obstacle to effective therapy with agents targeted toward individual factors and receptors. Identification of common mechanism(s) underlying expression of such a diversity of factors could guide the development of therapy using fewer selective agents. We previously observed that IL-8, VEGF and other cytokines are coexpressed and often vary concurrently in serum and supernatants from cell lines from different patients with head and neck squamous cell carcinoma (HNSCC),4-5 suggesting that these factors could be regulated by common signal pathways or transcription factors.

Examination of the regulatory region of many proinflammatory cytokines and proangiogenic factors reveals that they share common promoter sites for transcription factors such as nuclear factor-kappa-B (NF-κB) and/or activator protein-1 (AP-1), which can be activated by injury, cytokines and growth factors.6 We found that differences in expression of these cytokines in different cancers was often related to differences in constitutive activation of both NF-κB and AP-1.6 Inhibition of NF-κB activation by expression of a dominant negative inhibitor-kB or pharmacologic agents was found to inhibit expression of IL-8 and other proinflammatory and proangiogenic cytokines,7-10 as well as tumorigenesis and angiogenesis in vivo.7-9 Inhibition of activation of extracellular signal-regulated kinase (ERK) and AP-1 with antagonists of mitogen-activated/extracellular signal-regulated kinase (MEK) partially inhibited expression of both IL-8 and VEGF.10 These results provided evidence that at least 2 signal pathways upstream of NF-κB and AP-1 make important contributions to expression of these angiogenesis factors.

We have recently examined the contribution of several factors that may contribute to upstream signal activation of NF-κB and AP-1 and expression of IL-8 and VEGF. We found that IL-1α expressed by HNSCC promotes autocrine activation of NF-κB and AP-1, expression of IL-8 and cell survival.11 Expression of IL-1 receptor antagonist inhibited NF-κB reporter activity and IL-8 expression by 60–80%, indicating that IL-1α is a major contributor to constitutive activation of NF-κB and IL-8. HNSCC were also found to express c-MET, a receptor tyrosine kinase for hepatocyte growth factor/scatter factor (HGF/SF).12 Increased expression of HGF/SF was detected together with IL-8 and VEGF in serum of patients with HNSCC, and recombinant HGF and HGF from stromal fibroblasts was found to further induce IL-8 and VEGF expression by human HNSCC lines. Inhibitors of MEK and
the phosphotidyl inositol 3-kinase (PI3K) signal pathways partially involved in angiogenesis and cytokines in HNSCC. We also recently found that epidermal growth factor as well as IL-1α can induce activation of NF-κB and the murine IL-8 homologue growth regulated oncogene-1 (Gro 1, also known as KC). IL-1α and EGF induced activation of NF-κB and Gro-1 could be blocked by expression of a dominant negative IκBαM. These results suggested that members of the epidermal growth factor receptor (EGFR) family as well as cytokines such as IL-1α could promote expression of angiogenesis factors through activation of NF-κB and AP-1 in squamous cell carcinoma.

Overexpression and EGFR phosphorylation is detected in 90% of HNSCC and is also frequently detected in other major cancers of epithelial origin, including carcinomas of the lung, breast, prostate, ovary, and bladder. Increased expression of EGFR and its ligands in HNSCC has been associated with a poorer prognosis and been shown to promote malignant transformation and tumorigenesis in vitro and in vivo. EGFR has been reported to modulate several important mechanisms involved in tumor development, progression and therapeutic resistance, including angiogenesis. Antagonists of EGFR have been reported to inhibit IL-8 and VEGF, indicating that EGFR may directly or indirectly regulate coexpression of these angiogenesis factors. However, the intermediary signal pathways and transcription factors that activate these proangiogenic factors are unknown. EGFR can induce the downstream activation of several important intermediate signal transduction kinases, including Ras, phosphotydilinositol-3-kinase (PI3K), MEK and Janus kinase (JAK). These signal components in turn have been shown to be able to activate several signal pathways that activate transcription factors, such as AP-1, NF-κB and signal transduction and transcription factor-3 (STAT3).

In our study, we examined the effect of antagonists of EGFR, PI3K and MEK on NF-κB and AP-1 activation and IL-8 and VEGF expression in HNSCC cell lines UM-SCC-9 and 11B. We provide evidence that EGFR can induce NF-κB and AP-1 activation and IL-8 and VEGF expression in HNSCC and that antagonists of EGFR, PI3K and MEK have inhibitory activity against EGFR-induced activation of these transcription and cytokine factors. EGFR antagonists partially inhibited constitutive NF-κB and AP-1 activation and IL-8 and VEGF expression. The coactivation of these intermediary kinases and transcription factors by EGFR and other growth factor and cytokine receptors suggests that therapy targeting multiple receptors or common signal pathways may be necessary for optimal inhibition of angiogenesis and cancer therapy. Further molecular and clinical characterization of EGFR, MEK and PI3K as targets for therapeutic inhibition of oncogenic transcription factors NF-κB and AP-1 and angiogenesis factors in HNSCC and other cancers in which these transcription and cytokine factors are activated is warranted.

MATERIAL AND METHODS

Cell lines and tissue culture

The UM-SCC-9 and 11B HNSCC cell lines from the University of Michigan UM-SCC series were kindly provided by Dr. T. E. Carey (University of Michigan, Ann Arbor, MI) and described previously. The UM-SCC-9 I-13 line transfected with a dominant negative inhibitor-kappaBα alpha phosphorhbitity mutant (IκBαM) and the UM-SCC-9 C-13 line transfected with control vector were established, characterized and maintained as previously described. UM-SCC cell lines were maintained as monolayer cultures in Eagle’s minimal essential medium (EMEM) supplemented with 10% fetal bovine serum, 50 μg/ml penicillin/streptomycin and 2 mM glutamine (MEM complete). Normal keratinocytes were obtained from Clontech (Palo Alto, CA) and maintained in keratinocyte growth medium ( Gibco BRL, Grand Island, NY) supplemented with 5 ng/ml EGF and 50 μg/ml penicillin/streptomycin. All normal human keratinocytes used for experiments were used prior to six passages in culture.

Reagents and antibodies

Recombinant human epidermal growth factor (EGF), recombinant human transforming growth factor alpha (TGFα) and ELISA kits were purchased from R&D Systems (Minneapolis, MN). PD153035 is an EGFR tyrosine kinase inhibitor purchased from Tocris (Ballwin, MO). Clone 225 (C225) is a murine monoclonal EGF-specific antibody purchased from Calbiochem (Cat# GR13L, La Jolla, CA). The C225 isotype control, mouse IgG1 (Cat# 03000D), was purchased from PharMingen (San Diego, CA). LY-294002 is a PI3K inhibitor purchased from Biomol (Plymouth Meeting, PA), and U0126 is a MEK inhibitor obtained from Promega (Madison, WI). The BCA Protein Assay and Super Signal West Pico Chemiluminescent Detection kits were purchased from Pierce (Rockford, IL). Rabbit polyclonal antibodies for phospho-Akt (Ser473) (Cat# 9271), phospho-ERK1/2 (Thr202/Tyr204) (Cat# 9101), Akt (Cat# 9272), ERK1/2 (Cat# 9102) and horseradish peroxidase (HRP)-linked anti-rabbit IgG (α-rabbit-HRP) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) included: goat EGFR agrose conjugate (EGFR-AC, Cat# sc-03-AC), goat polyclonal EGFR antibody (Cat# sc-03-G), mouse monoclonal phospho-tyrosine (PY99) antibody (Cat# sc-7020), goat polyclonal p-EGFR (Tyr 1173) (Cat# sc-12351), rabbit polyclonal antibody for β-tubulin (Cat# sc-9104), Protein G PLUS Agarose (Cat# sc-2002), normal goat IgG (Cat# sc-2028) and anti-goat IgG-HRP (Cat# sc-2020). The anti-mouse IgG-HRP antibody (Cat# 170-6516) was from Bio-Rad (Hercules, CA). 7XAP-1 and 5XNF-κB-Luciferase reporter gene constructs were from Stratagene (La Jolla, CA). LipofectAMINE PLUS transfection reagents and precast Tris-Glycine gels (4–12% and 10%) were from Invitrogen (Carlsbad, CA).

Isolation of whole-cell lysates

Subconfluent cells were rinsed once with ice-cold PBS, scraped and lysed in 250 μL lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, 0.2 mM Na3VO4 and 0.2 mM PMSF) and passed three times through a 23-G needle. The lysates were centrifuged at 12,000g for 5 min at 4°C and the supernatant stored at −80°C. Total protein concentrations were determined using the Pierce BCA protein assay method.

Immunoblotting

Whole-cell lysates were mixed with Laemmli loading buffer (containing β-mercaptoethanol) and heated at 100°C for 5 min. The samples were loaded onto Tris-Glycine precast gels (4–12% gradient gels for EGFR Westerns and 10% gels for Akt and ERK1/2 Westerns) and electrophoresed at 1200 V. The proteins were transferred to 0.45 μm nitrocellulose membranes (Bio-Rad) for 90 min at 20V at room temperature using the Novex Gel Blot Module. Immunoblotting for phospho-EGFR, EGFR and β-tubulin was conducted according to Santa Cruz specifications. Specifically, primary antibodies were diluted 1:100 for phospho-EGFR and 1:1,000 for EGFR and β-tubulin in 5% nonfat powdered milk prepared in TBST and incubated with the nitrocellulose blots for 2 hr at 4°C. Secondary α-goat-HRP and α-rabbit-HRP antibodies were diluted 1:2,000 in 5% nonfat powdered milk prepared in TBST and incubated with the nitrocellulose blots for 1 hr at room temperature.

Immunoblotting for phospho- and nonphospho-Akt and ERK1/2 was conducted according to Cell Signaling protocol. Primary antibodies were diluted 1:1,000 and incubated with nitrocellulose blots overnight at 4°C and secondary antibodies were diluted 1:2,000 in 5% BSA prepared in TBST and incubated with blots for 1 hr at room temperature. Each blot was incubated with Pierce Super Signal West Pico substrate and exposed to Kodak X-OMAT film.
Immunoprecipitation

Total EGFR was immunoprecipitated from 500 μg of whole-cell lysate according to the Santa Cruz protocol. After the final wash, the pellets were resuspended in 5 μL 4X Laemmli whole-loading dye (containing β-mercaptoethanol), heated at 95°C for 3 min and loaded onto 4–12% gradient Tris-Glycine precast gels. The samples were electrophoresed and transferred to nitrocellulose as described above. The primary antibodies against phospho-tyrosine (PY99) and EGFR were diluted 1:1,000 in 5% nonfat powdered milk prepared in TBST and incubated with the nitrocellulose blots for 2 hr at 4°C. The secondary antibodies were diluted 1:2,000 (α-mouse-HRP for PY99 and α-goat-HRP for EGFR) in 5% nonfat powdered milk prepared in TBST and incubated with the blots for 1 hr at room temperature.

Transient transfection and luciferase reporter assays

UM-SCC-9 and 11B were seeded at 1–2 × 10^5 cells per well in 96-well culture plates. On the following day, the cells were co-transfected with AP-1 or NF-kB luciferase reporter gene constructs (0.1 μg/well) and pCMV-LacZ (0.005 μg/well) using 0.25 μL per well of LipofectAMINE and 0.5 μL per well PLUS reagent with minor modifications according to manufacturer’s protocol (Invitrogen, Carlsbad, CA). Three hours after transfection, medium was removed and replaced with complete MEM for 24 hr. The cells were preincubated with inhibitors for 24 hr and then stimulated with 5 ng/mL EGF for 18 hr. The cells were washed once with PBS and lysed in 25 μL Tropix lysis solution per well. Lysates were stored at −80°C until analysis. Luciferase activity was determined using a Dual Light Reporter Gene assay (Tropix, Bedford, MA) and a Wallac Victor2 1420 Multilabel Counter according to manufacturer’s instructions (Wallac, Gaithersburg, MD). β-galactosidase activity was determined to verify the reproducibility between quadruplicate transfections in all experiments.

Cytokine quantitation by ELISA

Cells were plated at 5 × 10^4 cells per well in 24-well culture plates. The next day, cells were preincubated with inhibitor for 1 hr and stimulated with 5 ng/mL EGF for 24 hr. Culture supernatants were harvested and centrifuged at 12,000g for 5 min at 4°C to pellet any debris. The supernatants were stored at −80°C until ELISA analysis. ELISA for IL-8 and VEGF was performed according to manufacturer’s protocol (R&D Systems).

Measurement of cell proliferation by MTT assay

A total of 5 × 10^3 UM-SCC-9 or 11B cells were plated in a 96-well microtiter plate and incubated overnight. Then the cells were washed twice with PBS and exposed to antagonists in medium at the concentrations indicated. Cell density was determined using an MTT cell proliferation assay (Boehringer Mannheim, Indianapolis, IN). MTT labeling reagent was added at days 1, 3 and 5 after treatment, and colorimetric O. D. were measured at 570 nm by a microplate reader (Biotek, Winooski, Vt).

Statistical analysis

Results from control and test conditions for reporter and ELISA experiments were compared using student’s t-test, and differences resulting in probability of error of p < 0.05 were accepted as statistically significant.

RESULTS

Expression of EGFR and inhibition of constitutive and EGF-inducible phosphorylation in HNSCC cell lines UM-SCC-9 and 11B by EGFR tyrosine kinase inhibitor PD153035

Overexpression of EGFR has been detected in more than 90% of HNSCC. To determine the expression of EGFR in HNSCC cell lines previously found to exhibit constitutive activation of NF-κB, AP-1 and proangiogenic cytokine genes, whole-cell lysates from 2 representative HNSCC cell lines, UM-SCC-9 and UM-SCC-11B, and normal human keratinocytes as a control were analyzed by Western blot analysis for EGFR expression. Figure 1a shows that EGFR is detected in UM-SCC-9, UM-SCC-11B and primary human keratinocytes. EGFR was detected in higher concentration in UM-SCC-9 and UM-SCC-11B when compared to primary human keratinocytes and when normalized to β-tubulin as a control, consistent with overexpression of EGFR observed in HNSCC in other studies. As a measure of EGFR activation, we examined EGFR tyrosine phosphorylation in keratinocytes, UM-SCC-9 and UM-SCC-11B cell lines. Total EGFR from whole-cell lysates was immunoprecipitated and analyzed for tyrosine phosphorylation using anti-phospho-tyrosine antibodies by Western blot. Figure 1a shows constitutive tyrosine phosphorylation of EGFR in both UM-SCC-9 and UM-SCC-11B but not in normal keratinocytes.

The effects of EGF and the well-characterized EGFR tyrosine kinase inhibitor PD153035 on phospho-Tyr EGFR and on total EGFR was determined. Figure 1b, Lane 1 shows constitutive tyrosine phosphorylation of EGFR in both UM-SCC-9 and UM-SCC-11B, and phosphorylation was further increased when cells were incubated with 5 ng/ml EGF (Fig. 1b, Lane 4). Basal and EGF-inducible phosphorylation of EGFR in UM-SCC-9 and UM-SCC-11B could be blocked by 1 and 5 μM EGFR kinase inhibitor, PD153035, in a dose-dependent manner (Fig. 1b, Lanes 2, 3 and 5,6). Blots were reprobed for total EGFR, demonstrating that the effects observed with EGF and PD153035 were not due to
modulation of total EGFR or unequal loading. The higher concentration of phospho-Tyr-EGFR detected and concentration of PD153035 needed for inhibition in UM-SCC-11B was observed in repeated experiments and is consistent with the higher level of constitutive activation of NF-κB and AP-1 and cytokine expression detected in this cell line relative to UM-SCC-9 (results below).4–6

Effects of EGF and PD153035 on activation of NF-κB and AP-1 and expression of IL-8 and VEGF in UM-SCC-9 and 11B

To investigate whether EGFR regulates functional activation of NF-κB and AP-1 and expression of IL-8 and VEGF, we examined the effects of EGF and the EGFR tyrosine kinase inhibitor PD153035 on basal and EGF-inducible activation of these transcription and proangiogenesis factors in the UM-SCC-9 and 11B cell lines. Luciferase reporter gene constructs under the control of multiple NF-κB and AP-1 binding sites were used for the functional analysis of NF-κB and AP-1 activity. As shown in Figure 2a, UM-SCC-9 and 11B exhibited basal NF-κB and AP-1 activity, and stimulation with EGF induced a 1.5–2-fold increase in reporter activity. Preincubation with PD153035 at concentrations that inhibited EGFR tyrosine phosphorylation in Figure 1b completely blocked EGF-inducible activation and resulted in greater than 50% inhibition in basal activation of both NF-κB and AP-1 (Fig. 2a, p < 0.05).

Since we previously showed that transcription factors NF-κB and AP-1 promote expression of proangiogenic cytokines in HNSCC,6–10 we examined the effect of EGF and PD153035 on IL-8 and VEGF production. As shown in Figure 2b, UM-SCC-9 and UM-SCC-11B constitutively secreted IL-8 and VEGF at low and high levels, respectively, consistent with previous studies.10 Stimulation with EGF increased concentrations of both IL-8 and VEGF. PD153035 completely blocked EGF-induced production and inhibited basal production of both factors by more than 50% (p < 0.05), consistent with the effects on NF-κB and AP-1 activity observed in Figure 2a.

Effects of anti-EGFR antibody C225 on NF-κB and AP-1 activation and IL-8 and VEGF production

Anti-EGFR antibodies specific for the EGFR binding domain have been shown to inhibit EGFR activation25 and expression of IL-8 and VEGF by immunohistochemistry.25–27 We tested the effect of C225 on NF-κB and AP-1 activation and IL-8 and VEGF production. Figure 3a shows that 2 μg/ml of C225, but not an isotype control antibody, strongly inhibited EGF-inducible NF-κB and AP-1 reporter activity in both cell lines (p < 0.05). C225 inhibited basal AP-1 activation approximately 50% (p < 0.05) but inhibited basal NF-κB activity by only approximately 20%, and this effect did not reach the level of statistical significance (p > 0.05). The effect of C225 on basal NF-κB and AP-1 reporter activity was not further increased by doubling the concentration of antibody to 4 μg/ml (data not shown). However C225 had an inhibitory effect on both EGF induced and constitutive IL-8 and VEGF production, similar to that of PD153035 (compare Figures 2b and 3b). No significant inhibition was observed with an isotype control antibody. Together, these data provide evidence that either PD153035 or C225 can inhibit EGF-induced activation of NF-κB and AP-1 and coexpression of IL-8 and VEGF in UM-SCC-9 and 11B. Both PD153035 and C225 inhibited constitutive expression of IL-8 and VEGF by UM-SCC-9 and 11B, although C225 had a greater effect on constitutive activation of AP-1 than upon NF-κB.

EGFR activates PI3K and MEK pathways in UM-SCC-9 and 11B

PI3K and MEK are important intermediary kinases downstream of growth factor receptors such as EGFR, and PI3K and MEK pathways have been implicated in activation of NF-κB and AP-1, respectively.29,30,32,33 PI3K has been reported to modulate phosphorylation of Akt or GSK-3β and activation of NF-κB,41–43 and MEK has been shown to modulate phosphorylation of MAPK ERK1/2 and activation of AP-1.10 To establish whether these signaling pathways are activated in HNSCC, we determined the basal state of phosphorylation of one PI3K substrate, Akt, and MEK substrate ERK1/2 in UM-SCC-9 and 11B. Figure 4, Lane 1 shows that Akt(473) and ERK 1/2 are constitutively phosphorylated in UM-SCC-9 and 11B. To determine whether EGFR directly regulates functional activation of these pathways, we examined the effect of EGF and the EGFR kinase inhibitor PD153035 on phosphorylation of Akt and ERK1/2. Stimulation with EGF increased phosphorylation of ERK1/2 and Akt in both cell lines (Fig. 4, Lane

![Figure 2](https://example.com/figure2.png)

**Figure 2**—PD153035 inhibits constitutive and EGF-inducible NF-κB and AP-1 activity and IL-8 and VEGF cytokine expression. (a) UM-SCC-9 and 11B were transiently transfected with NF-κB (upper panels) and AP-1 (lower panels) luciferase reporter constructs, preincubated with 0, 1 and 5 μM PD153035 (24 hr) and stimulated with 5 ng/mL EGF (18 hr). Lysates were harvested and analyzed from luciferase activity. Preincubation with PD153035 at concentrations that inhibited EGFR tyrosine phosphorylation in Figure 1b completely blocked EGF-induced activation and resulted in greater than 50% inhibition in basal activation of both NF-κB and AP-1 (Fig. 2a, p < 0.05). (b) Lysates were harvested from the same transfected cells, preincubated with PD153035 (1 hr) and stimulated with 5 ng/mL EGF (18 hr). Culture supernatants were harvested and analyzed for IL-8 and VEGF production by ELISA. Each value represents the mean concentration ± SD of duplicate samples from 1 of 3 independent experiments.
SCC-9 and SCC-11B. The translation of PI3K and MEK substrates Akt and ERK1/2 in UM-SCC-11B contributes to constitutive and EGF-inducible phosphorylation or integrity of the proteins. These results provide evidence that constitutive and EGF-inducible phosphorylation was not due to altered total expression of the proteins. To examine the contribution of PI3K and MEK to activation of the transcription factors and pro-angiogenic cytokines, we examined the effects of the well-characterized PI3K inhibitor LY-294002 and MEK inhibitor U0126 on substrate phosphorylation, reporter activity and cytokine production. In preliminary studies, we determined that 10 μM LY-294002 and 10 μM U0126 provided the optimal concentrations for inhibition of basal and EGF-induced phosphorylation of PI3K substrate Akt and MEK substrate ERK1/2. Figure 5a shows the effect of LY-294002 and U0126 on Akt and ERK1/2 phosphorylation in UM-SCC-11B. Figure 5a, Lanes 2,6 show that LY-294002 preferentially inhibited constitutive phosphorylation of Akt relative to ERK1/2. Conversely, Figure 5a, Lanes 3,7 show that U0126 preferentially inhibited phosphorylation of ERK1/2 when compared to Akt. Figure 5a, Lanes 4 and 8 show that the combination blocked both Akt and ERK. Thus, LY-294002 and U0126 preferentially inhibit PI3K and MEK, respectively.

The effect of the LY-294002 and U0126 on NF-κB and AP-1 activity and IL-8 and VEGF production in UM-SCC-11B is shown in Figure 5b. LY-294002 preferentially inhibited basal and EGF-inducible NF-κB activity, consistent with the preferential inhibition of upstream kinase PI3K. Conversely, U0126 significantly inhibited EGF-induced AP-1 activity and had no effect on NF-κB activity. U0126 had a greater inhibitory effect on inducible than on prior constitutive AP-1 activity during the course of the assay. Figure 5b also shows that combining LY-294002 and U0126 resulted in greater inhibition of constitutive and EGF-inducible NF-κB activity. In contrast, the combination of inhibitors only inhibited EGF-inducible AP-1 activity, consistent with the effect of U0126 alone. The effects of the PI3K and MEK inhibitors on production of IL-8 and VEGF are shown in Figure 5c. Basal and EGF-inducible IL-8 production was partially inhibited by both LY-294002 and U0126, consistent with dependence of IL-8 expression on both NF-κB and AP-1 activation, which we demonstrated previously by mutational analysis of the IL-8 promoter.6,11 However, U0126 had a greater inhibitory effect than LY-294002 on constitutive and EGF-inducible VEGF production. Figure 5c also shows that combining LY-294002 and U0126 had a greater inhibitory effect on cytokine production than either
Expression of a dominant negative mutant inhibitor-κB promotes EGF-inducible expression of both IL-8 and VEGF. EGF-inducible expression of IL-8, whereas MEK activation of PI3K activation of NF-κB inhibits inhibitor alone. Together, the findings above provide evidence that PI3K activation of NF-κB contributes primarily to basal and EGF-inducible expression of IL-8, whereas MEK activation of AP-1 promotes EGF-inducible expression of both IL-8 and VEGF.

**Effects of EGFR, MEK and PI3K antagonists on cell proliferation and viability**

The EGFR, MEK-AP-1 and PI3K-NF-κB signal pathways have been implicated in cellular proliferation and survival. To determine the effects of EGFR, MEK and PI3K antagonists on cell proliferation and/or survival, we cultured HNSCC lines with PD153035, U0126 and LY294002, and determined the effect on cell proliferation and viability. At concentrations where EGFR, PI3K and MEK antagonists inhibited downstream substrates and transcription factor reporter activity by 24 hr, significant inhibition of growth was observed from 3–5 days. At the highest concentrations, EGFR, PI3K and MEK antagonists had a negative effect on growth of UM-SCC-11B, whereas the effects on UM-SCC-9 were inhibitory. Negative growth in cultures was associated with the appearance of blebbing, fragmented and non-viable cells, consistent with decreased survival. These observations are consistent with previous data showing that UM-SCC-11B is relatively more sensitive than UM-SCC-9 to inhibition of NF-κB by transient transfection of dominant negative IκBαM. We conclude that EGFR, PI3K and MEK antagonists that inhibit NF-κB and AP-1 activation and cytokine expression can inhibit growth and/or survival of HNSCC.
DISCUSSION

In our study, we examined the effects of EGF and pharmacologic antagonists of EGFR, PI3K and MEK upon NF-κB and AP-1 activation and IL-8 and VEGF expression as outlined in the simplified model in Figure 8. We found that EGF stimulated and EGFR tyrosine kinase antagonist PD153035 inhibited EGFR phosphorylation and downstream coactivation of transcription factor NF-κB and AP-1 and expression of IL-8 and VEGF (Figs. 1, 2). The effects of anti-EGFR monoclonal antibody C225 on EGFR signaling and coactivation of these transcription and angiogenesis factors were also examined. C225 but not an isotype control antibody completely inhibited EGF-inducible NF-κB and AP-1 reporter activity and production of both cytokines (Fig. 3). However, while both PD153035 and C225 had similar effects on constitutive activation of AP-1 and expression of both angiogenesis factors, the anti-EGFR antibody C225 had weak if any inhibitory effect compared to PD153035 on constitutive NF-κB reporter activity, suggesting that PD153035 and C225 may have different effects downstream on NF-κB-dependent and -independent mechanisms that regulate constitutive cytokine expression. We also examined the effects of EGFR and selective inhibitors of PI3K and MEK on functional NF-κB and AP-1 reporter activity and IL-8 and VEGF expression in HNSCC. EGFR tyrosine kinase antagonist PD153035 inhibited downstream coactivation of PI3K and MEK signal transduction pathways as evidenced by inhibition of phosphorylation of PI3K substrate Akt and MEK substrates ERK1/2 (Fig. 4). We found that the MEK pathway is an important contributor to AP-1 activation, VEGF expression and coexpression of IL-8, whereas the PI3K signaling pathway preferentially contributes to NF-κB activation and IL-8 expression (Fig. 5). The inhibition of EGF-induced NF-κB reporter activity and IL-8 expression following inhibition of NF-κB by dominant negative IκBαM provided further direct molecular evidence that NF-κB contributes to EGF-induced expression of IL-8. EGFR, PI3K and MEK antagonists also inhibited growth or survival (Fig. 7). The data presented provide evidence that EGFR, PI3K and MEK pathways can contribute to NF-κB and AP-1 activation, IL-8 and VEGF expression and growth by HNSCC. Further molecular studies using dominant negative constructs and clinical studies using pharmacologic inhibitors of these signal mediators are warranted to determine optimal combination and efficacy of therapies targeting inhibition of NF-κB and AP-1 and angiogenesis factors in HNSCC and other cancers.

The incomplete inhibitory effects of both EGFR antagonists on constitutive NF-κB and AP-1 reporter activity are consistent with our previous studies in human and murine SCC, which indicate that IL-1α is another important factor that contributes to autocrine activation of NF-κB and AP-1 and particularly to expression of NF-κB-regulated cytokines huIL-8 and muGro 1. We recently found that IL-1α and EGF can both induce activation of NF-κB and expression of the murine IL-8 homologue growth regulated oncogene-1 (Gro 1, also known as KC) in murine SCC Pam 212.13 IL-1α- and EGF-induced activation of NF-κB and Gro-1 was attenuated by expression of a dominant negative IκBαM.13 These results indicated that cytokine IL-1α and EGF promote expression of Gro 1 through activation of NF-κB in murine SCC. In human HNSCC, differences in expression of IL-1α, a potential inducer of NF-κB and AP-1, were noted to correlate with differences in constitutive activation of NF-κB and AP-1 and expression of IL-8 in the low and high cytokine producers UM-SCC-9 and 11B,6 consistent with the hypothesis that IL-1α could regulate constitutive activation of these transcription and angiogenesis factors. IL-1α was shown to induce NF-κB and AP-1 activation, while mutations of the NF-κB, and to a lesser extent, the AP-1 promoter
EGFR ACTIVATION OF NF-κB, AP-1, IL-8 AND VEGF

sites, attenuated IL-1α-mediated IL-8 reporter gene activity. Expression of IL-1 receptor antagonist blocked autocrine activation of NF-κB and IL-8 cytokine expression by 60–80%, indicating that IL-1 is a major contributor to constitutive activation of NF-κB and IL-8 in HNSCC. Consistent with this, only a relatively minor inhibitory effect of approximately 20% on constitutive NF-κB activity was seen at the limits of detection using the EGFR receptor-specific antibody C225 in our study (Fig. 3). A greater effect on constitutive NF-κB activity was seen with PD153035, possibly indicating that this inhibitor has stronger inhibitory effects on EGFR, other EGFR family members or downstream kinases that contribute to activation of NF-κB.

Recently, evidence that EGFR and other EGFR family members contribute to activation of NF-κB in other cancers has been independently reported by several other laboratories, providing evidence that these findings are of broader relevance. Biswas et al. showed that EGFR is expressed and phosphorylated and contributes to NF-κB activation in human breast cancer cells. They showed that inhibition by an anti-EGFR antibody and by protein kinase C (PKC) inhibitor Go6976 resulted in inhibition of NF-κB p65 phosphorylation, transactivation of the NF-κB target gene cyclin D1 and decreased proliferation. Panietti et al. stably transduced Ba/F3 rat mammary tumor cells to overexpress human EGFR, Her-2/neu or other EGFR family member genes alone or in combination. Elevated NF-κB activity was observed in the EGFR gene transfectant, Ba/F3-1, as well as other transfectants with 2 EGFR family genes in combination. Habib et al. reported that EGFR activates NF-κB in a high EGFR-expressing breast cancer cell line, MDA-MB-468, but not in a low EGFR-expressing breast cancer cell line, MCF-7, and suggested that EGFR density is important for EGFR-induced NF-κB activation in the breast cancer cells. They also showed that Rat-1 fibroblasts do not respond to EGFR, whereas R1hER cells, a Rat-1 transfectant with highly expressed and phosphorylated EGFR, had constitutively activated NF-κB and responded strongly to exogenous EGF. Since EGFR, Her-2/neu, as well as IL-1 and other factors could contribute to activation of NF-κB in patients with HNSCC, it will be important to conduct further studies with genetic and pharmacologic antagonists to identify optimal proximal targets for antiangiogenesis and cytotoxic therapy of HNSCC.

Both EGFR antagonists and the combination of MEK and PI3K inhibitors had greater inhibitory effects on expression of proangiogenic cytokines than was observed or predicted by either NF-κB and AP-1-specific reporter activity alone. Greater inhibition at the level of gene transcription could result from synergistic interactions between NF-κB and AP-1. Evidence for synergistic interaction between NF-κB and AP-1 in activation of the IL-8 and other cytokine promoters has previously been reported. Several recent reports have also provided evidence for physical interaction between NF-κB and AP-1, and the resulting coactivation has been implicated in transformation. In addition, transactivation by NF-κB and AP-1 could be increased by activation of other factors involved in the promoter complex that may not be reflected by reporters containing limited consensus binding sequences. EGFR also activates other signal and transcription factor pathways that could modulate expression of these cytokines. EGFR has been shown to activate STAT3 in HNSCC. This pathway appears to play an important role in resistance to apoptosis, and recent evidence suggests it may contribute to VEGF expression in cardiac myocytes. Finally, we have not excluded the possibility that EGFR and IL-1 could also modulate additional posttranscriptional and posttranslational mechanisms that affect cytokine secretion. Determination of the basis for the differences between reporter and protein expression may provide additional insight and potential targets for therapy. Deletion and mutation studies of IL-8 and VEGF promoters are underway to identify additional transcription factors and signal pathways that may contribute to transcriptional regulation by EGFR, IL-1 and other growth factors.

We examined the role of PI3K and MEK pathways, which are 2 major pathways downstream of EGFR that can activate regulation of NF-κB and AP-1, respectively. EGFR tyrosine kinase antagonist PD153035 inhibited downstream coactivation of PI3K and MEK signal transduction pathways as evidenced by inhibition of phosphorylation of PI3K substrate Akt and MEK substrates ERK1/2 (Fig. 4). Using inhibitors of PI3K and MEK (Fig. 5) and a dominant negative IκBαM stably expressing cell line (Fig. 6), we obtained evidence that these intermediate signal pathway components can mediate EGFR signal activation of NF-κB and AP-1 and expression of IL-8 and VEGF in HNSCC. Combining inhibitors against both PI3K and MEK pathways had a similar effect on NF-κB and AP-1 activation and cytokine production as inhibition of EGFR in UM-SCC-11B cells (Figs. 2, 5), indicating that these 2 signaling pathways are likely to be of major importance in EGFR-mediated regulation of these proangiogenic factors in HNSCC. MEK inhibitor U0126 was found to preferentially inhibit constitutive and EGF-inducible ERK1/2 phosphorylation, AP-1 activation and production of both IL-8 and VEGF (Fig. 5). These results are consistent with the previous findings by others that U0126 can block ERK1/2 phosphorylation and AP-1 activation and with our previous results indicating that U0126 can block TNF-α-inducible ERK1/2 phosphorylation, AP-1 activation and IL-8 and VEGF production in HNSCC. In contrast, we found that PI3K inhibitor LY-294002 preferentially inhibited constitutive and EGF-inducible NF-κB transactivation and IL-8 production by UM-SCC-11B cells (Fig. 5). Consistent with this, Biswas et al. have recently shown that LY-294002 can inhibit EGFR-induced activation of NF-κB and proliferation in breast cancer cells, and Pianetti et al. showed that breast cancer cells overexpressing Her-2/neu activate
NF-κB via the PI3K/Akt pathway. In the latter study, pharma-
cole and dominant negative mutant inhibitors of PI3K, Akt and 
casein kinase II also inhibited EGFR/Her-2/neu-induced transac-
tivation of NF-κB, providing evidence for the role of PI3K and Akt 
in transactivation of NF-κB.

The intermediary pathways that contribute to PI3K-mediated 
activation of NF-κB and transactivation of IL-8 remain to be 
defined. Although we observed that EGFR antagonist PD153035 
inhibited constitutive Akt phosphorylation, induction of Akt phos-
phorylation by EGF was modest (Figs. 4, 5) compared to the fold 
increases in activation of NF-κB and expression of cytokines 
detected (Fig. 5). Although Akt phosphorylation served to provide 
evidence for EGFR-mediated PI3K activation and specificity of 
PI3K and MEK inhibitors in HNSCC, EGFR and PI3K can induce 
other pathways besides Akt that mediate NF-κB and cytokine gene 
activation. Recently, GSK-3β has been reported as another inter-
mediary kinase important in NF-κB activation. Also recently, 
Romieu-Mouré et al. have also shown that overexpression of 
human kappαB kinase and casein kinase II downstream of EGFR 
may also contribute to activation of NF-κB in human breast 
cancers. Because multiple kinases may modulate transactivation 
of NF-κB, it will be important in future studies to further deter-
mine the role of PI3K, Akt, NIK, IKK and CK2 in NF-κB activation 
in HNSCC by genetic dominant negative expression studies.

We did perform further genetic analysis to confirm the role of 
NF-κB in mediating EGFR-induced activation of NF-κB and IL-8 
expression and to confirm that PI3K inhibitor had a greater effect 
on NF-κB and IL-8 expression than on AP-1 activation and VEGF 
expression. Genetic analysis using a stably transfected dominant 
negative mutant IκBα cell line, UM-SCC-9-I13, confirmed that 
inhibition of NF-κB activation inhibited EGFR-induced production 
of IL-8 but not VEGF (Fig. 6), indicating that NF-κB and AP-1 
activation have different effects on IL-8 and VEGF expression. 
In contrast, Huang et al. observed that IκBαM stable transfection 
blocked constitutive IL-8 and VEGF production in human ovarian 
cancer cells and we have observed inhibition of both the murine 
IL-8 homologue Gro 1 and VEGF in murine SCC following 
inhibition of NF-κB activation by proteasome inhibitor. It will 
be important to determine if these apparent differences in NF-κB-
regulated expression of IL-8 and VEGF in different tumor lines are 
due to direct or indirect effects of activation of NF-κB on the 
VEGF promoter, since the VEGF promoter lacks an apparent 
NF-κB DNA binding site.

The EGFR, MEK-AP-1 and PI3K-NF-κB have been individu-
ally implicated in cellular proliferation and survival, and EGFR, PI3K and MEK 
activators partially inhibited growth or survival of HNSCC (Fig. 7). 
PI3K, which activates NF-κB, appeared to be more toxic than the 
EGFR and MEK inhibitors (Fig. 7). We previously showed that inhibition of NF-κB activation by expression of a dominant negative IκB or proteasome inhibitor 
PS-341 resulted in a marked decrease in survival of human and 
murine SCCs, indicating NF-κB is an important survival pathway 
in SCC. Inhibition of NF-κB and EGFR has been shown to 
further sensitize SCC to apoptosis induced by cytotoxic radiation 
treatment. We did not observe strong antiproliferative ef-
fects or further radiation sensitization with MEK inhibitors (Fig. 7 
and C. Bancroft and J.B. Mitchell, unpublished observations).

This data suggest that antagonists of EGFR and NF-κB activation 
may have both antiproliferative and cytotoxic activity, especially 
when used in combination with radiation, which is a primary 
modality in treatment of HNSCC.

In summary, in our study we have provided evidence linking 3 
critical cancer-promoting mechanisms in HNSCC, which include: 
(i) activation of EGFR and its downstream signaling pathways; 
(ii) constitutive and inducible activation of NF-κB and AP-1; 
and (iii) autonomous proangiogenic factor production. These data provide evidence for a link between EGFR activation, coactivation of NF-κB and AP-1, and coexpression of IL-8 and VEGF in 
HNSCC. Our data indicate that inhibition of NF-κB and AP-1 
activation and proangiogenic factor expression may be an im-
portant mechanism underlying the activity of anti-EGFR antibodies 
and tyrosine kinase inhibitors in HNSCC and other cancers that 
overexpress EGFR. In cancers in which EGFR and other signals 
such as IL-1 activate these pathways, inhibition of NF-κB and 
AP-1 activation may block common pathways important for 
expression of these angiogenesis factors and other early genes that 
promote malignant behavior. Our recent observations in human 
and murine SCC provide evidence for the view that multiple 
signals can activate common signal pathways that promote expres-
sion of genes that mediate malignant behavior. Determining 
the mechanisms underlying activation of multiple pathways and 
genes may bring comprehensive understanding and improved 
strategies for the use of therapeutic agents needed for the success-
ful treatment of cancer.

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