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Activated Extracellular Signal-regulated Kinases: Association with Epidermal Growth Factor Receptor/Transforming Growth Factor α Expression in Head and Neck Squamous Carcinoma and Inhibition by Anti-Epidermal Growth Factor Receptor Treatments[1]

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ABSTRACT

The expression of the activated mitogen-activated kinases/extracellular signal-regulated kinases (ERKs) ERK1 and ERK2 was characterized in 101 human head and neck squamous carcinoma specimens. Activated ERK1/2 were detected at different levels in the majority of these tumors, as assayed by immunostaining with an antibody specific for the dually phosphorylated and activated ERK1 and ERK2. ERK1/2 activation levels were higher in tumors with advanced regional lymph node metastasis ($P = 0.048$) and in relapsed tumors ($P = 0.021$). The expression of epidermal growth factor (EGF) receptor ($P = 0.037$), transforming growth factor α (TGF-α; $P < 0.001$), and HER2 ($P = 0.066$; positive trend) correlated with activation of ERK1/2. In a multivariate analysis, both TGF-α ($P < 0.0001$) and HER2 ($P = 0.045$) were independently correlated with ERK1/2 activation. In turn, activation of ERK1/2 was associated with a higher Ki-67 proliferative index ($P = 0.002$). In EGF receptor-dependent model cells (A431 and DiFi), a specific EGF receptor tyrosine kinase inhibitor ("Iressa"; ZD1839) and a chimeric anti-EGF receptor antibody ("Cetuximab"; C225) inhibited ERK 1/2 activation at concentrations that inhibited autocrine cell proliferation. In patients on treatment with C225, the activation of ERK1/2 in skin, an EGF receptor-dependent tissue, was lower compared with control skin. Parallel changes were seen in keratinocyte Ki67 proliferation indexes in skin from C225-treated patients. Taken together, these studies provide support for a role of activation of ERK1/2 in head and neck squamous carcinoma and a correlation with EGF receptor/TGF-α expression. The inhibition of ERK1/2 activation *in vitro* and *in vivo* by compounds targeting the EGF receptor points to the interest of ERK1/2 as potential surrogate markers of EGF-receptor signaling in clinical therapeutic studies.

INTRODUCTION

The EGF[5] receptor is a M_r 170,000 plasma membrane glycoprotein composed of an extracellular ligand-binding domain, a transmembrane lipophilic segment, and an intracellular protein kinase domain with a regulatory COOH-terminal segment (1). After ligand binding, EGF-receptor dimerization occurs, which results in high-affinity ligand binding, activation of the intrinsic protein tyrosine kinase activity, and tyrosine autophosphorylation (1). These events lead to activation of a cascade of biochemical and physiological responses that are involved in the mitogenic signal transduction of cells (2). Extensive preclinical studies (2, 3) have shown that these downstream signaling transduction cascades regulate multiple cellular processes such as proliferation, differentiation, survival, and transformation.

Several lines of evidence support the EGF receptor as a target for therapy of head and neck carcinomas. EGF receptor and one of its ligands, TGF-α, are overexpressed in the majority of head and neck tumors (Ref. 4 and references therein), and this overexpression correlates with a poor clinical outcome. Furthermore, inhibitors of receptor function such as MAbs and tyrosine kinase inhibitors have antiproliferative effects on EGF receptor-expressing cancer cells (5–8). Recently (9–11), clinical studies have demonstrated activity of anti-EGF receptor therapies in patients with advanced malignancies, and studies are currently under way with these agents in patients with head and neck carcinomas.

As these agents move into the clinic, a better understanding of the EGF receptor-dependent pathways *in vivo* and their pattern of expression/activation would be of interest for at least two reasons. First, they may be of assistance in predicting the subset of EGF receptor-positive tumors that will benefit from therapy; second, downstream signaling transduction molecules may prove to be useful surrogate markers of complete receptor blockade. This latest point is particularly relevant with this novel type of agents because an optimal biological dose (*i.e.*, a dose resulting in complete receptor inhibition) would be preferred to the maximally tolerated dose that is being used with conventional nontargeted chemotherapeutic agents (12).

A major signaling route of EGF receptor is the Ras-Raf-MAPK pathway (2). Activation of Ras initiates a multistep phosphorylation cascade that leads to the activation of MAPKs (13). The MAPKs ERK1 and ERK2 are activated by dual phosphorylation on a tyrosine and a threonine residue by dual specificity kinases. ERK1/2 subsequently regulate cell transcription and have been linked to cell proliferation, survival, and transformation in laboratory studies (13). Elevated levels of MAPK activation in tumor tissues compared with their corresponding non-neoplastic tissues have been reported recently (14–21) in several human tumors, although it remains uncharacterized in head and neck carcinomas.

In the present study, we have analyzed in a large series of head and neck squamous carcinomas the expression of activated ERK1/2 and their relationship with EGF receptor/TGF-α expression and proliferation in a clinical setting. Activated ERK1/2 were assessed by immunostaining with an antibody specific for the dually phosphorylated and activated MAPKs ERK1 and ERK2 (phospho-p44/42 MAPK; Ref. 22). We have found that activated ERK1/2 were present at different levels in the majority of tumors, and expression levels were correlated with EGF receptor/TGF-α expression and with the tumor proliferative

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[5] The abbreviations used are: EGF, epidermal growth factor; MAPK, mitogen-activated kinase; ERK, extracellular signal-regulated kinase; TGF, transforming growth factor; MAb, monoclonal antibody; FBS, fetal bovine serum; T, primary tumor stage; N, regional lymph node stage; TNM, Tumor-Node-Metastasis.

index. We then explored whether the level of activated ERK1/2 expression could be used as a marker of EGF-receptor function inhibition and antiproliferative effects of anti-EGF receptor-targeted therapies. Using two human tumor cell lines that have extensively been characterized to be EGF receptor-dependent, we observed a marked correlation between cell growth suppression and inhibition of ERK1/2 activation with two anti-EGF receptor agents currently in clinical trials, the tyrosine kinase inhibitor ZD1839 (Iressa[6]) and the MAb C225 (Cetuximab[7]). In addition, we show in skin, a well-characterized EGF receptor-dependent tissue (23–27), from patients treated with C225 that the activation of ERK1/2 was lower compared with control skin. This decrease in activated ERK1/2 was accompanied by a decrease in keratinocyte proliferation. Taken together, these studies show that activated ERK1/2 are present and correlated with EGF receptor/TGF-α in head and neck tumors and that they are potential surrogate markers of EGF-receptor activation or inhibition that should be further explored in the clinic.

MATERIALS AND METHODS

Tissues. Archival tissue specimens from patients with head and neck squamous carcinoma were retrieved from the Department of Pathology (Vall d'Hebron University Hospital, Barcelona, Spain) following institutional guidelines. A series of 101 primary tumors was assayed for the expression of activated ERK1/2 and the assessment of the potential relationships between activated ERK1/2, EGF-receptor signaling components (EGF receptor, TGF-α, and HER2 receptor), and proliferative index (Ki-67). We also analyzed paired primary tumors and relapsed tissue specimens from 19 patients of this series who suffered a relapse. Overall, 120 specimens were analyzed from the 101 patients. Demographic and treatment data had been recorded previously. Clinical tumor staging was performed according to the TNM classification of malignant tumors (28). Slides were reviewed for tumor grade.

We also studied 10 control skin samples (*i.e.*, from patients not treated with EGF-receptor inhibitors) from our pathology archives and skin samples from four head and neck cancer patients participating in clinical trials with C225. Two biopsies from these C225-treated patients were from rashes that developed during treatment with C225 plus radiation therapy. The other two patients were on treatment with C225 plus cisplatin, and biopsies were from macroscopically normal skin. In one of them, a paired baseline (before C225 treatment) skin biopsy was also available. A separate written informed consent was obtained for these skin biopsies.

Compounds and Antibodies. EGF receptor tyrosine kinase inhibitor ZD1839 (kindly provided by AstraZeneca; Ref. 7) and anti-EGF receptor MAb C225 (kindly provided by Imclone Systems; Ref. 5) were used for *in vitro* assays. The primary antibodies used were: rabbit polyclonal phospho-p44/42 MAPK (Thr202/Tyr204) antibody to activated ERK1/2 (New England BioLabs Inc., Beverly, MA), rabbit polyclonal ERK1/2 antibody to total ERK1/2 (New England BioLabs Inc.), mouse antibody to external domain of EGF receptor (BioGenex, San Ramon, CA), mouse MAb Ab2 to TGF-α (Oncogene Science, Cambridge, MA), mouse MAb CB-11 to HER2 (BioGenex), and mouse MAb B126.1 to Ki-67 (Biomeda Corp., Foster City, CA). Two negative control rabbit polyclonal immunoglobulins (BioGenex; Santa Cruz Biotechnology Inc., Santa Cruz, CA) and a negative control mouse monoclonal immunoglobulin (BioGenex) were also used.

Immunohistochemistry. All of the specimens had been fixed in 10% buffered neutral formalin, dehydrated, and paraffin embedded. Immunostaining was performed using 4-μm tissue sections placed on poly-L-lysine-coated glass slides. After deparaffinization in xylene and graded alcohols, epitope retrieval was performed. Target retrieval for activated ERK1/2, TGF-α, HER2, and Ki-67 was made in 10 mM EDTA buffer (pH 8) for 10 min in a microwave at 600 W. Epitope retrieval for EGF receptor was made by pepsin digestion for 10 min. After epitope retrieval, endogenous peroxidase was blocked by immersing the sections in 0.03% hydrogen peroxide for 15 min. Slides were washed for 5 min with PBS. Incubations with primary antibodies were made at room temperature for 1 h at the following dilutions: activated ERK1/2 1:50, total ERK1/2 1:50, EGF receptor 1:30, TGF-α 1:50, HER2 1:20, and Ki-67 1:1 (prediluted form). The peroxidase-labeled polymer conjugated to goat antirabbit (activated ERK1/2 and total ERK1/2) or antimouse (EGF receptor, TGF-α, HER2, and Ki-67) method was used to detect antigen-antibody reaction (DAKO EnVision+ System; DAKO Corporation, Carpinteria, CA) for 30 min at room temperature. Sections were then visualized with 3,3′-diaminobenzidine as a chromogen for 5 min and counterstained with Mayer's hematoxylin. Slides were washed in tap water, dehydrated, and mounted with glass coverslips. Positive controls were sections of a tissue specimen previously found to be positive for the antigen of interest. The negative controls consisted of duplicate sections of the same specimens in which the primary antibody had been excluded and replaced with PBS or negative control immunoglobulins. The controls for activated and total ERK1/2 were stained with the same amount of antihuman polyclonal rabbit IgG instead of the primary antibodies. The controls for EGF receptor, TGF-α, HER2, and Ki-67 were stained with the same amount of antihuman monoclonal mouse IgG instead of the primary antibodies. Representative tumor sections were identified on a light microscope with an ocular magnification of $\times$25. To score a tumor cell as positive for a given marker, complete membrane staining was required for EGF receptor and HER2, cytoplasmic or membrane staining for TGF-α, and nuclear staining for activated ERK1/2 or Ki67. The percentage of stained tumor cells was scored from these sections in 10 high-power fields ($\times$400), and the average percentage of tumor cell staining for each antibody was calculated (by F. R.). Tumors with $>$1% of tumor cells staining for a given marker were considered positive for such marker (29). Grading of positivity ranged from a score of 1% to 100%. This scoring was used for statistical correlation analysis between the studied markers and was not intended to provide a clinical cutoff value. The same scoring system was used for skin specimens. Scoring was blinded to the clinical data.

Cells and Monolayer Growth Assay. Two human tumor cell lines that overexpress the EGF receptor, A431 vulvar squamous carcinoma cells and DiFi colon adenocarcinoma cells (5, 6), were used in this study. We confirmed the overexpression of the EGF receptor in both A431 and DiFi cells by immunocytochemistry, although we did not detect HER2 staining. Cells were grown in monolayer culture with DMEM:Ham's F-12 (1:1) with 10% FBS at 37°C and 5% CO_2. For monolayer growth assay, cells were seeded in 6-well culture plates (model 3046; Falcon, Lincoln Park, NJ) at 10^4 cells/cm^2. The next day, cells were changed to medium containing 0.5% FBS for 18 h, and ZD1839 or C225 was added at various concentrations to the cultures. After 72 h, cells were washed once with PBS, harvested with 0.1% trypsin-1 mM EDTA in PBS, and counted with a Coulter counter.

Western Immunoblotting. Western immunoblotting was performed as reported previously (6) with minor modifications. Cells were seeded in parallel and under the same conditions as for monolayer growth assays. The monolayers were then exposed to the indicated concentrations of ZD1839 or C225. After 2 h, the medium was removed, the cells were washed twice with cold PBS, and the monolayer was scraped into 1 ml of ice-cold lysis buffer [50 mM HEPES (pH 7.0), 10% glycerol, 1% Triton X-100, 5 mM EDTA, 1 mM $MgCl_2$, 25 mM NaF, 50 μg/ml apronitin, 50 μg/ml leupeptin, 0.5 mM orthovanadate, and 1 mM phenylmethylsulfonyl fluoride]. The lysates were transferred to a clean microfuge tube, placed on ice for 15 min, and centrifuged for 10 min at 14,000 rpm. The supernatant was transferred to a clean microfuge tube, and protein concentration was determined. Protein extracts (50 μg) were boiled in Laemmli buffer 2$\times$ and resolved on a 10% SDS-polyacrylamide gel, before transferring to a nitrocellulose membrane. Membranes were blocked in Tris-buffered saline [50 mM Tris-HCl (pH 7.4), 150 mM NaCl] with 5% nonfat milk overnight at 4°C and then incubated with antibodies to total ERK1/2 (1:1000) or to activated ERK1/2 (1:1000) for 2 h at room temperature. Subsequently, membranes were washed three times for 5 min each in the same solution and then incubated for 60–90 min with an antirabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Freiburg, Germany). Subsequently, membranes were vigorously washed four times for 5 min each in the same solution, followed by a quick incubation using an ULTRA SuperSignal chemiluminescence substrate (Pierce, Rockford, Illinois).

Immunocytochemistry. Cells were seeded on positive-charged slides placed in culture plates under the same conditions used for monolayer growth assays and Western blot assays. On the next day, cells were changed

[6] "Iressa" is the property of the AstraZeneca group of companies.
[7] "Cetuximab" is the property of Imclone Systems Inc.

to medium containing 0.5% FBS for 18 h, and then ZD1839 or C225 was added at the indicated concentrations to the cultures for 2 h. After 2 h, the slides were removed from the culture plates, and cells on the slides were fixed in 2% paraformaldehyde solution at 4°C for 30 min and then treated with 1% BSA and 0.1% saponine to permebealize cells at room temperature for 30 min. Nonspecific protein reactivity was blocked with Protein Block (BioGenex) for 10 min, and endogenous peroxidase activity was blocked in hydrogen peroxide solution at 0.01% for 10 min. The antibodies used were the same as for the immunohistochemistry assays. Specimens were incubated for 1 h at room temperature with the antibody to total ERK1/2 (dilution, 1:100) or to activated ERK1/2 (dilution, 1:100). Immunodetection of antigens was made with Super Sensitive Immunodetection System (BioGenex). To detect the binding of antibody to antigen, slides were incubated with biotinylated antirabbit-immunoglobulins for 20 min at room temperature and then with horseradish peroxidase-labeled streptavidin complexes for 20 min at room temperature. Slides were then visualized using 3–3′-diaminobenzidine as a chromogen for 1 min. Then, specimens were dehydrated and coverslipped in nonaqueous permanent mounting media. Positive and negative controls were included in each staining run.

Statistical Methods. All of the statistical analyses were carried out using SPSS Data Analysis Program version 9.0. A Kolmogorov-Smirnov test was applied to determine whether a variable followed a normal distribution. Pearson's correlations were made between continuous variables (*i.e.*, percentage of squamous carcinoma cell staining) that followed a normal distribution in our series of 101 tumors, and nonparametric Spearman's correlations were made when one or both of the covariables did not follow a normal distribution. Activated ERK1/2, EGF receptor, and Ki67 followed a normal distribution and TGF-α did not. In the case of HER2, because most of the tumors were zeros ($n = 82$), correlation was calculated only for those tumors that were HER2 positive ($n = 19$) and followed a normal distribution. A multivariate regression analysis that included all of the cases was planned to assess the independent value of the association between all of the EGF receptor-signaling members analyzed that may interact *in vivo* (*i.e.*, EGF receptor, TGF-α, and HER2) and activated ERK1/2, assessed on a continuous scale. Paired samples were analyzed using the *t* test for paired samples. The relationship between categorical variables and continuous variables was analyzed by the Fisher's exact test or Mann-Whitney *U* test. All of the statistical tests were conducted at the two-sided 0.05 level of significance.

RESULTS

Expression of Activated ERK1/2 in Human Head and Neck Squamous Carcinoma. One-hundred and one primary head and neck squamous carcinoma specimens were analyzed for activated ERK1/2. The clinicopathological characteristics of the 101 patients were: mean age, 57 years (range, 31–78); male, $n = 86$; female, $n = 15$; lip and oral cavity, $n = 85$; pharynx, $n = 16$; T_1, $n = 1$; T_2, $n = 23$; T_3, $n = 12$; T_4, $n = 55$; T_x, $n = 10$; N_0, $n = 37$; N_1, $n = 21$; N_2, $n = 26$; N_3, $n = 10$; N_x, $n = 7$; TNM Stage II, $n = 8$; III, $n = 17$; IV, $n = 68$; unknown, $n = 8$; well differentiated, $n = 24$; moderately, $n = 46$; poorly differentiated, $n = 31$ (Table 1). At sampling, only 11 patients had received prior therapy, which consisted of chemotherapy and radiation.

The antibody used in the present study detects ERK1/2 only when catalytically activated by phosphorylation at Thr202 and Tyr204, and its specificity has been shown previously (*e.g.*, see Ref. 22). Positive nuclear staining for activated ERK1/2 was seen in some basal and parabasal epithelial cells of oral mucosa and in cells from minor salivary glands in adjacent non-neoplastic squamous epithelium. Activated ERK1/2 was also detected in endothelial cells, smooth muscle cells, Schwann cells, and fibroblasts. These nonmalignant cells had a weak cytoplasmic pattern and occasionally intense nuclear staining. Lymphocytes and plasma cells did not stain. Endothelial cells and lymphocytes were considered as internal positive and negative controls for each slide, as used by others (Fig. 1*A*; Ref. 19). A subset ($n = 20$) of tumors was analyzed with an antibody to total ERK1/2.

Table 1 *Patient demographics*

Characteristic	No. of patients[a]
Mean age yrs (range)	57 (31–78)
Sex	
Male	86
Female	15
Site	
Lip and oral cavity	85
Pharyngx	16
Primary tumor	
T_1	1
T_2	23
T_3	12
T_4	55
T_x	10
Regional lymph nodes	
N_0	37
N_1	21
N_2	26
N_3	10
N_x	7
TNM Stage	
II	8
III	17
IV	68
Unknown	8
Histopathological grade	
Well differentiated	24
Moderately differentiated	46
Poorly differentiated	31

[a] Numbers represent total number of patients for each characteristic (except for age). Because the total number of patients was 101, no percentages are given.

Diffuse cytoplasmic staining was seen with this antibody to total ERK1/2 in the majority of epithelial and stromal cells. Nuclear staining for total ERK1/2 was observed in some tumor cells located in areas that had tumor cells with nuclear staining for activated ERK1/2 in adjacent tissue slides (Fig. 1*B*). This differential localization of total ERK1/2 staining, mainly in the cytoplasm if phospho-ERK1/2-negative and nuclear if phospho-ERK1/2-positive, is most likely attributable to the activation of ERK1/2 that involves their translocation from the cytoplasm to the nucleus and represents further proof of the specificity of the antibodies used (13). Replacement of the primary antibody by the same amount of negative control polyclonal rabbit IgGs (two negative controls from different sources) did not show staining (Fig. 1, *C* and *D*).

The percentage of squamous carcinoma cells with nuclei staining for activated ERK1/2 varied among tumors (Table 2). In head and neck squamous carcinoma cells, staining for activated ERK1/2 was seen in the nucleus, and in general, a less intense cytoplasmic staining was seen (Fig. 1*A*). Nuclear staining was predominantly seen at the advancing margins of the tumor. In 6 of the 101 head and neck carcinoma specimens analyzed, a frozen tissue fragment of the same tumor specimen that had been paraffin-embedded was available. A Western blot analysis of activated ERK1/2 in those six samples showed a good relationship between the scored percentage of positive tumor nuclei staining and Western blot assay results (data not shown). Higher levels of activated ERK1/2, measured by the percentage of tumor nuclei cell staining in every specimen, were associated with a more advanced N stage (N_{0-2} *versus* N_3; Mann-Whitney *U* test; $P = 0.048$). Poorly differentiated tumors had higher levels of activated ERK1/2 than did well- or moderately differentiated tumors, but the difference did not have statistical significance ($P = 0.104$). No trends for associations were seen between activated ERK1/2 and T stage, sex, age, or tumor site. In 19 patients who relapsed after initial therapy, higher levels of activated ERK1/2 in the relapsed specimens were detected as compared with their corresponding primary tumor specimens (Fig. 1, *E–F*; paired *t* test; $P = 0.021$).

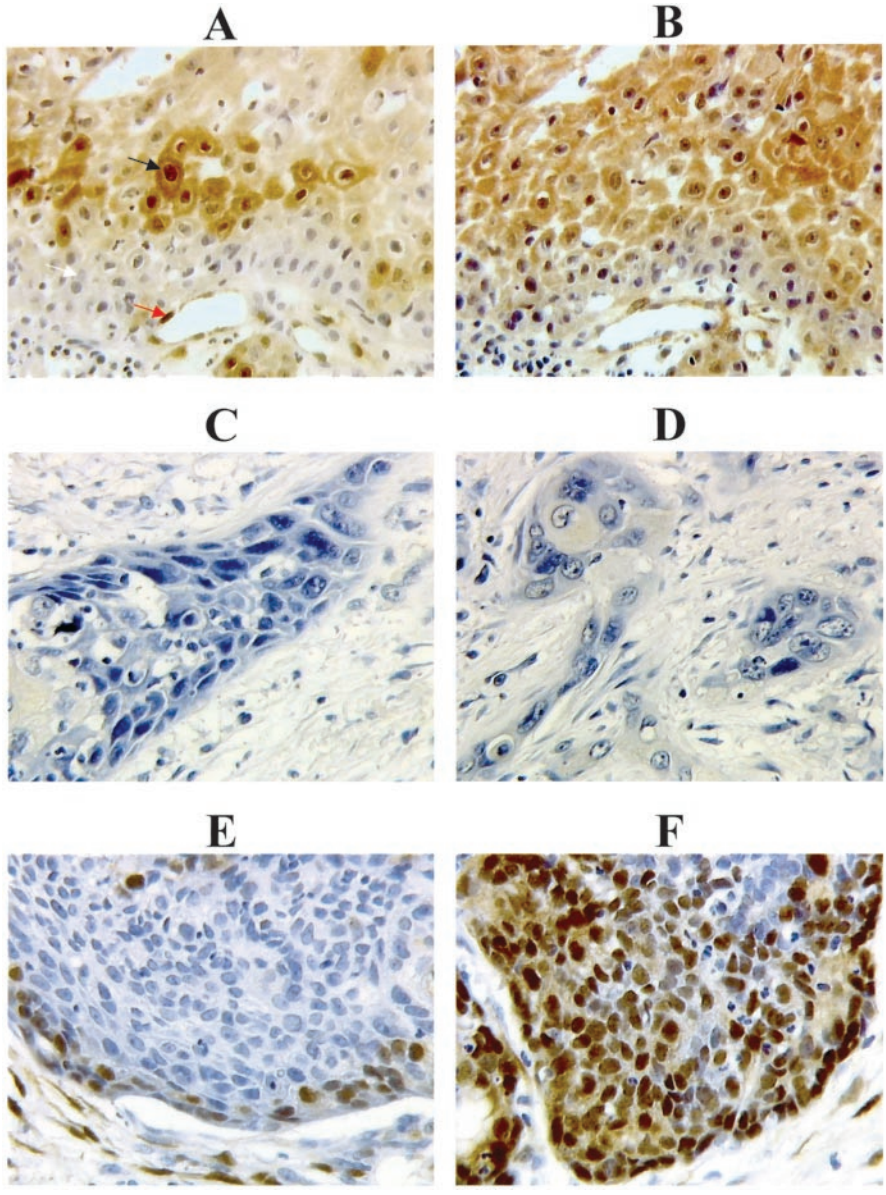

Fig. 1. Activated ERK1/2 in head and neck squamous carcinoma. *A*, a representative slide from a head and neck squamous carcinoma specimen stained with an antibody specific for the dually phosphorylated and activated ERK1/2. A subset of squamous carcinoma cells, mainly located in the periphery of the tumor nest, was stained with a predominantly nuclear pattern (*black arrow*). Lymphocytes (*white arrow*) and endothelial cells (*red arrow*) were used as internal negative and positive controls. *B*, an adjacent section to the one in *A* was stained with an antibody to total (phosphorylation state-independent) ERK1/2. As expected, the majority of squamous carcinoma cells exhibited cytoplasmic staining, whereas nuclear staining was more limited. In general, nuclear staining for total ERK1/2 was observed in tumor cells located in those areas that had nuclear staining for activated ERK1/2 in adjacent tissue slide. *C* and *D*, replacement of the antibody to activated ERK1/2 by the same amount of negative control polyclonal rabbit IgGs [two negative controls from different sources; rabbit polyclonal immunoglobulins from BioGenex (*C*) or from Santa Cruz Biotechnology, Inc. (*D*)] did not show staining in a tumor specimen that was positive for activated ERK1/2. *E* and *F*, a pair of a primary (*E*) and relapsed (*F*) head and neck carcinomas from the same patient stained with the antibody to activated ERK1/2 showing increased levels of activated ERK1/2 in the relapsed specimen.

Association between Activated ERK1/2 and Expression of EGF Receptor-signaling Family Members. Because ERK1/2 can be activated by EGF receptor signaling (13), we determined the relationship between activated ERK1/2 and expression of EGF receptor-signaling members, as measured by the percentage of tumor cells with positive staining for each antibody. The molecules analyzed were the EGF receptor, the EGF receptor ligand TGF-α, and the closely related HER2 receptor (Table 2). TGF-α was chosen because it is considered the predominant EGF receptor ligand expressed in malignancy (30). HER2 was included because this receptor also signals through ERK1/2 and is the preferred partner for EGF receptor dimerization (2), although it is detected in a limited subset of head and neck carcinomas (Ref. 31; Table 2). A significant relationship was observed between activated ERK1/2 and EGF receptor ($n = 101$; Pearson, $r = 0.21$; $P = 0.037$) and activated ERK1/2 and TGF-α ($n = 101$; Spearman, $r = 0.37$; $P < 0.001$; Fig. 2, *A–B*). In the case of HER2, because most of the tumors were negative (Table 2), the correlation between HER2 levels and ERK1/2 activation was calculated only for those tumors that were HER2 positive ($n = 19$). This analysis showed a trend to a positive association between HER2 and ERK1/2 activation levels (Pearson, $r = 0.43$; $P = 0.066$; Fig. 2, *A* and *C*). A linear multivariate analysis that included all of the cases was made to assess the independent value of the association between all of the EGF receptor-signaling members analyzed that may interact *in vivo* (*i.e.*, EGF receptor, TGF-α, and HER2) and activated ERK1/2. In this analysis, both TGF-α ($P < 0.0001$) and HER2 ($P = 0.045$) were independently associated with activated ERK1/2. EGF receptor expression lost its statistical significance in the multivariate analysis.

Table 2 *Immunostaining for activated ERK1/2, EGF receptor, TGF-α, HER2, and Ki-67 in 101 primary head and neck squamous carcinomas*

Percentage tumor cell staining[a]	Activated ERK1/2[b]	EGF receptor[b]	TGF-α[b]	HER2[b]	Ki-67[b]
0	9	16	5	82	0
1–10	21	4	1	3	2
11–25	48	8	2	6	25
26–50	22	17	15	8	54
>50	1	56	78	2	20

[a] Staining results were grouped in the indicated ranges of percentage of tumor cell staining for each antibody for purposes of data presentation.

[b] Numbers represent total number of tumors for each percentage of tumor cell staining for each antibody. Because the total number of specimens was 101, no percentages are given.

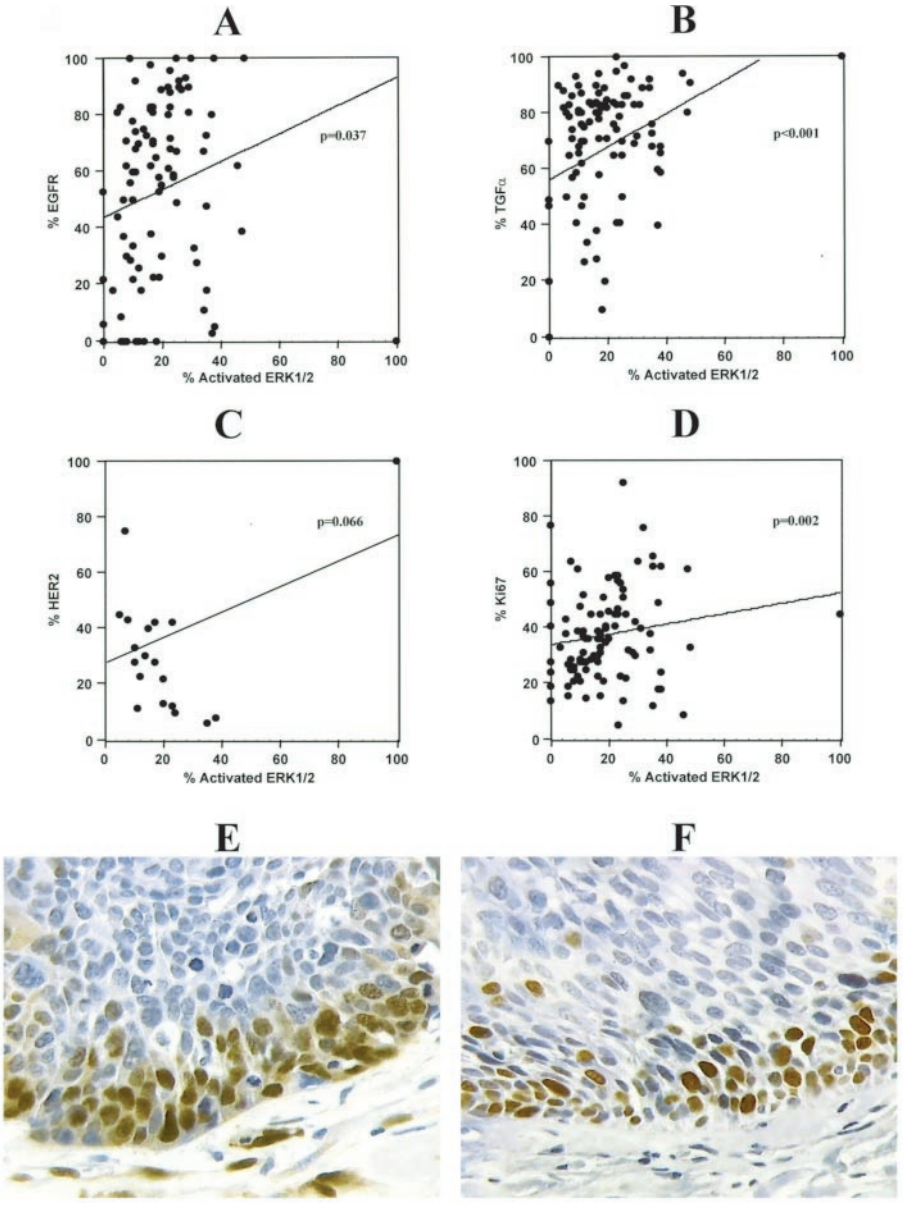

Fig. 2. Graphic representation of the correlations between the percentage of nuclei tumor cells staining for activated ERK1/2 and the percentage of tumor staining for EGF receptor (*A*), TGF-α (*B*), HER2 (*C*), and the proliferation marker Ki-67 (*D*) in head and neck carcinomas analyzed. *E*, a representative slide from a head and neck squamous carcinoma specimen stained with an antibody specific for the dually phosphorylated and activated ERK1/2 predominantly showing staining of cells in the periphery of the tumor. *F*, an adjacent section to the one in *E* was stained with an antibody to Ki67 and also shows a peripheral pattern of nuclear tumor cell staining.

Association between Activated ERK1/2 and Proliferative Index. Activation of ERK1/2 drives cell proliferation (13); therefore, we determined whether a correlation between activated ERK1/2 and proliferation exists in head and neck tumors. Ki-67, a nuclear proliferation-associated antigen present only in proliferating cells, was assessed immunohistochemically to measure cell proliferation rates (Table 2 and Fig. 2, *D* and *F*; Ref. 32). A significant correlation was observed between activated ERK1/2 and Ki-67 staining ($n = 101$; Pearson, $r = 0.30$; $P = 0.002$; Fig. 2*D*). Further supporting the association, the pattern of staining for both activated ERK1/2 and Ki-67 was predominantly located in the periphery of the tumors, a finding that may be related to the highest level of tumor proliferation at the edges (Fig. 2, *E* and *F*). The EGF receptor family-signaling members analyzed did not correlate with the proliferative index.

EGF Receptor Tyrosine Kinase Inhibitor ZD1839 and Anti-EGF Receptor MAb C225 Induce Suppression of ERK1/2 Activation at Doses Resulting in Growth Inhibition. To further define the possible role of activated ERK1/2 as a marker of EGF receptor activation and of the antiproliferative effects of EGF receptor-targeted therapies, a series of studies were performed *in vitro*. For these studies, we used two human cell lines, A431 and DiFi, that have high levels of EGF receptor expression and that have been extensively characterized by us and others (5, 6) to be EGF receptor-dependent. In these model cells, we analyzed the ability of the EGF receptor tyrosine kinase inhibitor ZD1839 (7, 9) and of the chimeric anti-EGF receptor MAb C225 (5, 12) to inhibit autocrine growth and activation of ERK1/2. To evaluate whether a correlation exists between concentrations of ZD1839 or C225 that inhibited growth and ERK1/2 activation, cell cultures driven by endogenous (autocrine) ligand were incubated with increasing concentrations of each compound. For Western blot assays, cells were incubated in the presence of compound for 2 h and then lysed. For proliferation assays, cells were incubated in the presence of compound for 72 h and then counted. Both ZD1839 (Fig. 3, *A* and *B*) and C225 (Fig. 3, *C* and *D*) inhibited the growth of these cells at concentrations of drug that can be achieved in patients. The effects on A431 cells and DiFi cells were similar but not identical, because DiFi cells were more sensitive to the growth inhibitory effects of the compounds analyzed and the degree of inhibition of ERK1/2 activation was greater in DiFi cells. Regardless of this, the concentrations of ZD1839 or C225 that caused a

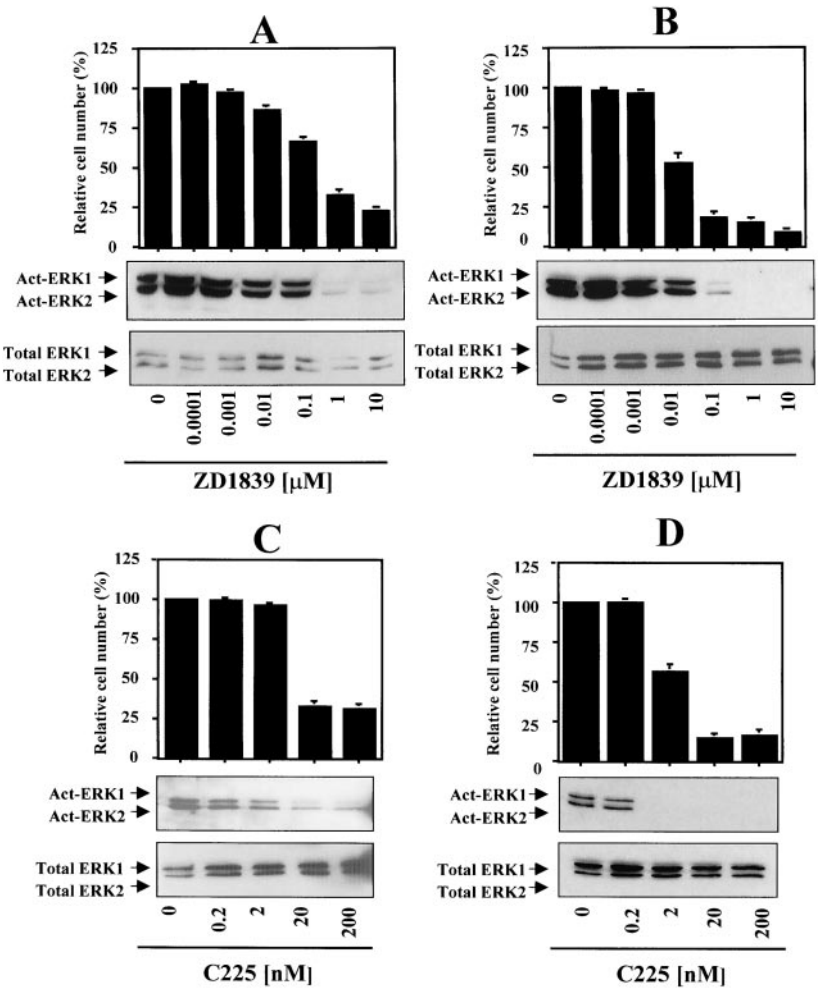

Fig. 3. The relationship between concentrations of the EGF receptor tyrosine kinase inhibitor ZD1839 or the chimeric anti-EGF receptor MAb C225 that inhibit autocrine tumor cell proliferation and concentrations that inhibit ERK1/2 activation in squamous A431 cells and in DiFi adenocarcinoma cells. *A*, A431 cells exposed to ZD1839. *B*, DiFi cells exposed to ZD1839. *C*, A431 cells exposed to C225. *D*, DiFi cells exposed to C225. Proliferation is expressed as the relative cell number calculated from the percentage of the cell number in untreated cultures of triplicate experiments (+SE) for every treatment point. At every treatment point, activated ERK1/2 (*act-ERK1/2*) and total (phosphorylation state-independent) ERK1/2 were analyzed by Western blot as indicated in "Materials and Methods."

decline in cell proliferation coincided with concentrations that reduced or abolished endogenous ERK1/2 activation in both cell lines, as assayed by Western blot with the activation state-specific antibody (Fig. 3). These effects on ERK1/2 activation were unrelated to down-regulation of total (phosphorylation state-independent) ERK1/2 proteins, because their levels did not decrease by treatment (Fig. 3).

Differential Cellular Distribution of ERK1/2 As a Function of Activation. We showed that EGF receptor inhibitors markedly reduced ERK1/2 activation and that this effect was not attributable to total ERK1/2 down-regulation (Fig. 3). Next, we studied *in vitro* whether a differential cellular distribution of ERK1/2 exists as a function of activation, as suggested from the observations in tumor samples. A series of immunocytochemical analyses were performed using the A431 (Fig. 4*A*) and DiFi cells (Fig. 4*B*) under the same culture conditions used for the proliferation and Western blot assays. We used immunocytochemistry to provide subcellular localization of total and activated ERK1/2 *in situ*. The cells were directly seeded in immunocytochemistry slides placed on culture plates. This approach avoids the steps of cell harvesting and centrifugation that would have been needed if cells were grown directly in culture plates and that might affect cell biology and morphology.

In cultures driven by endogenous (autocrine) ligand, both A431 and DiFi cells exhibited nuclei staining for activated (phosphorylated) ERK1/2 and for total (phosphorylation-independent) ERK1/2. Staining for activated phospho-ERK1/2 was predominantly nuclear, suggesting that an important fraction of ERK1/2 is basally phosphorylated and localized in the nuclei. Upon treatment with either ZD1839 or C225, activated phospho-ERK1/2 staining markedly decreased and total ERK1/2 staining shifted to a mainly cytoplasmic localization. These results suggested that treatment with anti-EGF receptor compounds prevents phosphorylation of ERK1/2 and results in the presence of nonphosphorylated, mainly cytoplasmic ERK1/2. Taken together, these data pointed out that the predominant cellular distribution of ERK1/2 varies as a function of activation, mainly nuclear for activated (phosphorylated) ERK 1/2 and mainly cytoplasmic for nonphosphorylated ERK1/2.

The Anti-EGF Receptor MAb C225 Inhibits ERK1/2 Activation and Proliferation in Patients. Our observations in EGF receptor model cell lines indicated a tight association between inhibition of growth by anti-EGF receptor compounds (C225 and ZD1839) and inhibition of ERK1/2 activation, leading to the concept that activated ERK1/2 might be a useful surrogate marker of activity of these compounds. To assess whether an anti-EGF receptor compound could also reduce ERK1/2 activation in patients, we assayed activated ERK1/2 in skin specimens from controls *versus* patients treated with C225. We chose to study skin because the basal layer of the epidermis has high levels of EGF receptor expression and the receptor plays a critical role in the regulation of epidermal biology (23–27). Furthermore, C225 may cause skin acneiform rashes, suggesting that this skin toxicity could be an indirect indicator of receptor targeting by C225 (12), and skin is easily accessible to biopsies.

We confirmed the expression of EGF receptor in epidermal and hair follicle keratinocytes in all of the control and experimental (from patients treated with C225) skin specimens analyzed and found a

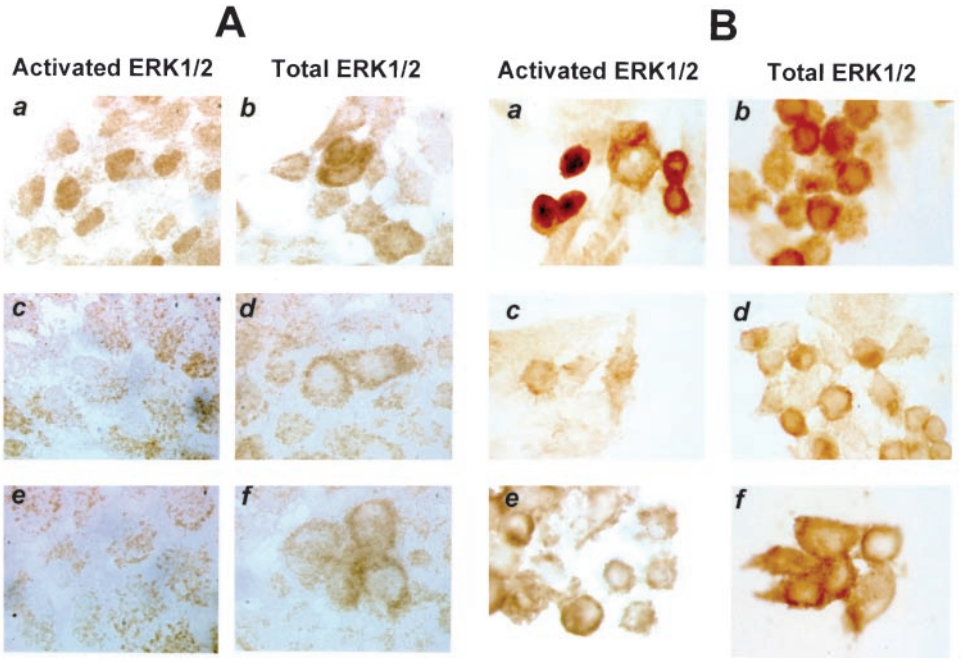

Fig. 4. Differential cellular distribution of ERK1/2 as a function of activation. *A*, A431 cell cultures driven by endogenous (autocrine) ligand without additions (*a* and *b*), with ZD1839 (10 μM; *c* and *d*), or with C225 (200 nM; *e* and *f*). *B*, DiFi cell cultures driven by endogenous ligand without additions (*a* and *b*), with ZD1839 (10 μM; *c* and *d*), or with C225 (200 nM; *e* and *f*). In cell cultures driven by endogenous ligand, many cells exhibited staining for activated (phosphorylated) ERK1/2, predominantly in the nuclei (*a*). Staining for total (phosphorylation-independent) ERK1/2 was strong in the cytoplasm and was less intense in general in the nuclei (*b*). After treatment with ZD1839 (*c*) or C225 (*e*), nuclei staining for activated ERK1/2 was markedly reduced or absent. In parallel, nuclear staining for total ERK1/2 was markedly reduced or absent [compare clear or "empty" nuclei in treated cells (*d* and *f*) with the stained nuclei in untreated cells (*b*)] and concomitantly shifted to a mainly cytoplasmic localization. For every condition, activated ERK 1/2 (*a*, *c*, and *e*) and total ERK1/2 (*b*, *d*, and *f*) staining were analyzed by immunocytochemistry as indicated in "Materials and Methods." Slides are shown in ×1000 magnification.

higher level of EGF receptor expression in keratinocytes of the basal layer of the epidermis (data not shown). Then, we characterized by immunohistochemistry the expression of activated ERK1/2 in 10 normal skin specimens using the same phospho-specific antibody to ERK1/2 that we used for the studies performed in clinical head and neck tumors and in tumor cells lines. In these control skin specimens (*i.e.*, from patients not treated with an EGF receptor inhibitor), activated ERK1/2 was mostly expressed in the nuclei of keratinocytes of the basal layer of the epidermis and in the outer root sheath of the hair follicles (Fig. 5, *A* and *B*), colocalizing with the population of proliferating, undifferentiated keratinocytes, where the EGF receptor is expressed at highest levels (Ref. 27 and data not shown). The percentage of keratinocytes with nuclei staining to activated ERK1/2 was scored in interfollicular epidermis in 10 high-power fields (×400). Hair follicles were qualitatively analyzed when present, but no scoring was conducted because many samples lacked them. In these 10 control samples, the percentage of basal keratinocytes staining for activated ERK1/2 was 25 ± 7% (mean and SD).

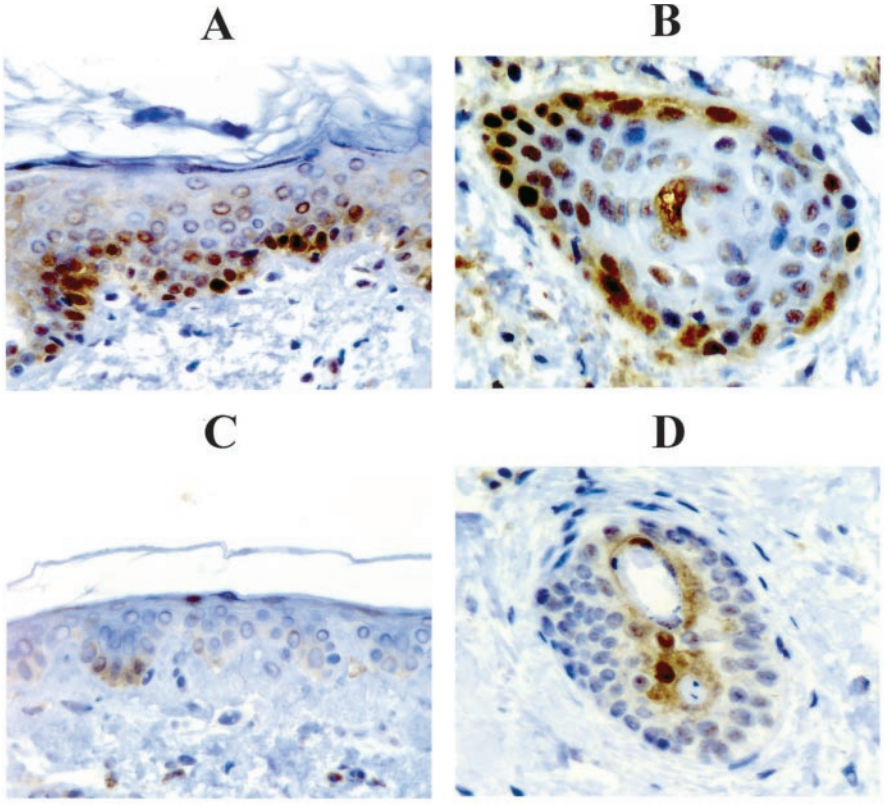

Fig. 5. Levels of activated ERK1/2 in paired pre- and on-C225 therapy skin biopsies. In normal human skin, activated ERK1/2 are expressed in interfollicular epidermis, mainly in the basal layer (*A*), and hair follicle, mainly in outer root sheath keratinocytes (*B*). During C225 treatment, the expression of activated ERK1/2 declined both in interfollicular epidermis (*C*) and in hair follicle keratinocytes (*D*; original magnification, ×400).

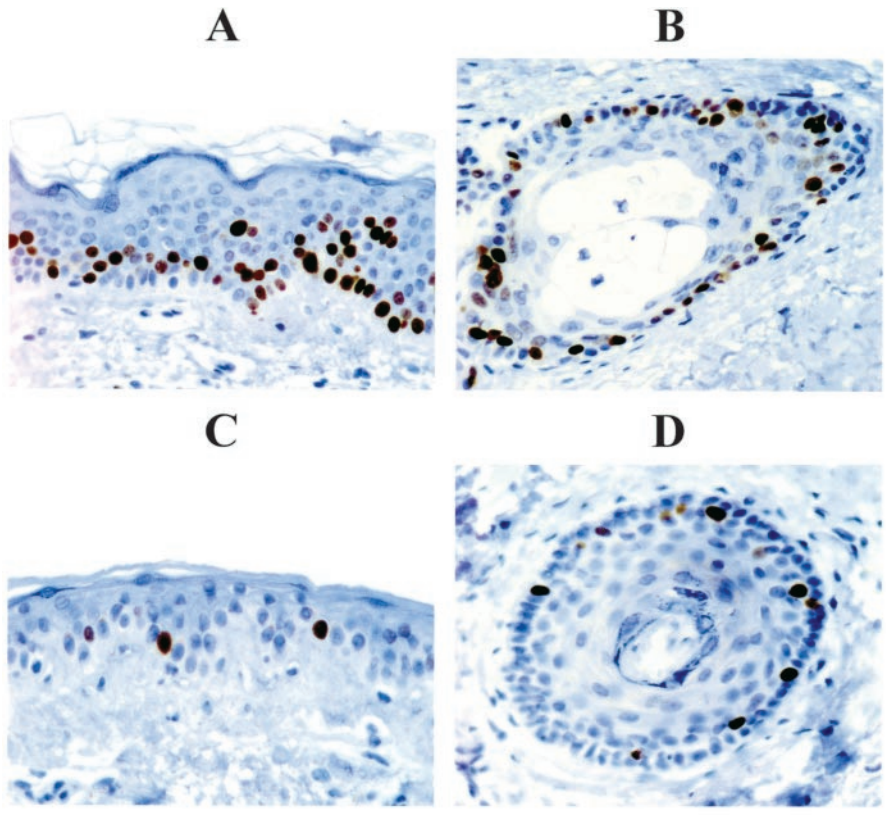

Fig. 6. Expression of the Ki67 proliferation markers in paired pre- and on-C225 therapy skin biopsies. In normal human skin, Ki67 is expressed in interfollicular epidermis, mainly in the basal layer (*A*), and hair follicle, mainly in outer root sheath keratinocytes (*B*). During C225 treatment, keratinocyte proliferation declined both in interfollicular epidermis (*C*) and in hair follicles (*D*; original magnification, ×400).

We then studied the expression of activated ERK1/2 in skin biopsies from patients that were on treatment with C225. In these patients, the schedule of C225 treatment consisted of a loading i.v. C225 dose of 400 mg/m^2, followed by 250 mg/m^2 i.v. weekly. We have shown previously (12) that patients treated with C225 at dose levels ≥200 mg/m^2 achieve sustained serum antibody concentrations above 200 nmol/liter, a concentration high enough to result in optimal antitumor activity in preclinical models. Therefore, we should be able to demonstrate C225 inhibition of ERK1/2 activation in these treated patients if the antibody inhibits EGF receptor signaling *in vivo*. Two patients were on treatment with C225, and radiation and skin biopsies were taken from skin rashes that developed during therapy in nonirradiated areas. The other two patients were on treatment with C225 plus cisplatin, and skin biopsies were taken from macroscopically normal skin. In one of them, a baseline skin biopsy was also available. Overall, in the four on-therapy biopsies, there were low levels of activated ERK1/2 expression in keratinocytes of the basal layer of the epidermis (Fig. 5*C*). The average percentage of positive basal keratinocytes from the four C225-treated patients was 5 ± 2% compared with an average 25 ± 7% positive basal keratinocytes from 10 control specimens. There was also an evident reduction of activated ERK1/2 in the outer root sheath of the hair follicle, as compared with the expression seen in control skin specimens (Fig. 5*D*). In a patient that underwent serial pre- and on-treatment skin biopsies, the expression of activated ERK1/2 was clearly reduced during C225 treatment (*i.e.*, 20% pre-C225 *versus* 3% on-C225; Fig. 5).

On the basis of the correlation between ERK1/2 activation and Ki67 proliferation index in our series of head and neck tumors and on the association between growth inhibition and inhibition of activated ERK1/2 in EGF receptor-dependent cell lines, we hypothesized that the reduced levels of ERK1/2 activation observed in skin from patients treated with C225 should be accompanied by a reduced proliferation rate. To study this possibility, we scored the percentage of basal keratinocytes with Ki67 staining in control *versus* C225-treated skin biopsies. The Ki-67 staining colocalized with activated ERK1/2; *i.e.*, mainly in basal layers and in the outer root sheath (Fig. 6, *A* and *B*). We observed a reduced keratinocyte proliferation index in skin biopsies taken during C225 treatment [$n = 4$; Ki67 proliferation index, 7 ± 4% (average and SD)] *versus* control specimens [$n = 10$; Ki67 proliferation index, 32 ± 12% (average and SD); Fig. 6*C*]. Inhibition of activated ERK1/2 was also seen in hair follicles (Fig. 6*D*). In the patient that underwent serial pre- and on-treatment skin biopsies, keratinocyte proliferation was also clearly reduced during C225 treatment (*i.e.*, 25% pre-C225 *versus* 2% on-C225; Fig. 6). This finding of a parallel effect of C225 treatment in patients on ERK1/2 activation and on proliferation in a human EGF receptor-dependent tissue further supports the notion of a biological effect of C225 on receptor downstream processes *in vivo*.

Overall, these findings indicate that EGF receptor blockade inhibits ERK1/2 activation and proliferation in patients in an EGF receptor-dependent tissue and provides rationale for the use of activated ERK1/2 in additional clinical studies with anti-EGF receptor compounds as a surrogate marker of biological activity in tumor biopsies.

DISCUSSION

The goal of this study was to characterize *in vivo* the pattern of expression of activated ERK1/2 in head and neck tumors and its relationship with expression of the upstream EGF receptor, HER2 receptor, and the ligand TGF-α. We chose to study ERK1/2 because of its critical role in the EGF receptor downstream-signaling pathway (2, 13), the known expression of total ERK1/2 in head and neck tumors (33), and the availability of reagents to study their level of activation in tumor samples at a cellular level. The levels of expression of activated ERK1/2 correlated with a higher nodal state and a higher proliferative rate and were increased in tumor relapses. We

found that the levels of activated ERK1/2 correlated with EGF receptor, TGF-α, and HER2 expression. In addition, inhibition of ERK1/2 activation in EGF receptor-dependent model cell lines with two different anti-EGF receptor therapies correlated tightly with growth inhibition, supporting its potential role as a surrogate marker *in vivo* of EGF receptor inhibition. We also showed that activation of ERK1/2 was lower in skin, an EGF receptor-dependent tissue (23–27), from patients treated with C225 compared with control skin, and this decrease in activated ERK1/2 was accompanied by a decrease in keratinocyte proliferation.

In head and neck squamous carcinoma, overexpression of total ERK1/2 had been observed (33), but the expression of the activated forms, which are necessary for EGF receptor-dependent signaling processes, was yet uncharacterized. We now show in a series of 101 primary head and neck squamous carcinomas that activated ERK1/2 is expressed in the nuclei of tumor cells in the majority of specimens, as determined by immunohistochemistry in paraffin-embedded specimens with an antibody that recognizes only the phosphorylated (activated) ERK1/2 (see "Results" and Ref. 22). This antibody allows the assessment of the activation state of ERK1/2 *in situ* and provides subcellular resolution of their nuclear translocation. We observed higher levels of ERK1/2 activation in tumors from patients with more advanced lymph node disease and in relapsed specimens compared with their corresponding primary tumors. These findings indicate that ERK1/2 are commonly activated in head and neck tumors and suggest they have a role in their malignant progression.

In our series of head and neck tumors, the levels of activated ERK1/2 were linked to the expression of EGF receptor-signaling members, further supporting that the Ras-Raf-MAPK pathway is activated by the EGF receptor *in vivo*. Increased levels of activated ERK1/2 were significantly correlated with the expression of the EGF receptor and TGF-α. In a multivariate analysis, TGF-α, but not the EGF receptor, independently associated with ERK1/2 activation, suggesting that autocrine/paracrine stimulation of the EGF receptor by ligand may be required for ERK1/2 activation. Perhaps, in head and neck tumors the presence of TGF-α may be required in addition to the almost universal expression of the EGF receptor to activate the receptor tyrosine kinase activity and drive critical downstream events, because it would be predicted from laboratory studies showing that genetically induced overexpression of EGF receptor ligands leads to cell transformation in the presence of the receptor (3). Taking into consideration that ERK1/2 may be activated by several pathways, the significant correlations observed between EGF receptor ($P = 0.037$) and TGF-α ($P < 0.001$) with activated ERK1/2 points to an important role of the EGF receptor in activating ERK1/2 in many head and neck tumors. The correlation coefficients (r) were less than 0.5 ($r = 0$ would be lack of correlation; $r = 1$ would be an absolute correlation in every single case), suggesting that ERK1/2 may also be activated by other receptors or downstream molecules in certain head and neck tumors. One example suggested in the present study was HER2, which in a multivariate analysis was associated with activated ERK1/2 independently of the EGF receptor and TGF-α. In the case of HER2, as expected (31), the number of HER2-overexpressing tumors was low. However, in those tumors that overexpressed HER2, a trend to a positive correlation between HER2 levels and ERK1/2 activation was found. When included in a multivariate analysis, HER2 was independently correlated with activation of ERK1/2. This finding may reflect that HER2 receptor activation leads to ERK1/2 activation in head and neck tumors or, alternatively, that HER2 availability for heterodimerization with EGF receptor is rate-limiting for EGF-mediated signal transduction and proliferation (34). However, the low rate of HER2 positivity detected in this series suggests that HER2 may play a role in a limited number of head and neck cancers. It is possible that other receptors or Ras mutations could lead to activation of ERK1/2, because some specimens had high levels of activated ERK1/2 without high levels of any of the EGF receptor family members analyzed. Conversely, in some tumors with high levels of one or more of the EGF receptor family members analyzed, the level of activated ERK1/2 was low or even undetected. In such tumors, it is possible that other EGF receptor-signaling transduction pathways, such as JAK/STAT or phosphatidyilinositol-3-kinase, are preferred instead (2, 35).

A few studies have addressed the expression of activated MAPK in nonsquamous tumors and its potential clinical/biological correlations. In prostate cancer, high levels of activated MAPK were observed in high-grade and advanced-stage tumors, suggesting elevated Ras signaling in advanced disease (19). In glial tumors, the presence of activated MAPK was also reported, and among these tumors, oligodendrogliomas showed an increase in the number of cells with MAPK activation with malignant progression (20). In renal cell carcinoma, MAPK activation correlated with MAPK kinase activation and Raf-1 activation (16). In hepatocarcinomas, a relationship was reported between ERK1/2 activation and the transcription factor c-Fos and cyclin D1 levels (17). In breast cancer, activated MAPK was associated with poor prognosis and decreased sensitivity to endocrine therapy and with the expression of phosphorylated-jun, a transcription factor activated by MAPK (21). In this series of breast adenocarcinomas, activated MAPK was correlated with the expression of EGF receptor, but not with TGF-α, which adds support, in a different histological tumor type, to our present finding of a link between activation of MAPK and EGF receptor expression in clinical tumors.

In the present study, we also report a significant correlation between activated ERK1/2 and the proliferative index, assessed by using Ki-67 as a marker. It is likely, however, that other pathways, such as the JAK/STAT or phosphatidyilinositol-3-kinase mentioned above (2, 35), also regulate proliferation in head and neck cancers. The pattern of staining for activated ERK1/2 and Ki-67 was predominantly at the tumor edges, suggesting that the EGF receptor may be predominantly activated in these areas. Preliminary experiments in a limited number of specimens with an antibody to the activated form of the EGF receptor suggest this might be the case.[8]

Additionally, our findings point to a potential role for activated ERK1/2 as surrogate markers of EGF receptor activity that may be useful to characterize *in vivo* the optimal biological dose of anti-EGF receptor treatments. To give further support to this later proposal, we studied *in vitro* the ability of the EGF receptor tyrosine kinase inhibitor ZD1839 (7) or the chimeric anti-EGF receptor MAb C225 (5), currently in clinical development (9–11), to affect growth and ERK1/2 activation in EGF receptor-dependent model cell lines. The results of these studies showed that the concentrations of ZD1839 or C225 that inhibited proliferation driven by endogenous (autocrine) ligand were the same that inhibited ERK1/2 activation. These effects were seen at drug concentrations that can be achieved in patients and that inhibit EGF receptor phosphorylation in cultured cells (5, 7). A greater inhibition of ERK1/2 activation was seen in DiFi cells compared with A431 cells, which is in agreement with the requirement of EGF receptor activation for both cell cycle progression and prevention of apoptosis in DiFi cells and a role of MAPK in survival (13, 36). In concordance with our findings, in TGF-α/HER2 bigenic mice treated with the EGF receptor tyrosine kinase inhibitor AG1478, regression of mammary tumors was associated with abolishment of constitutive MAPK activity (37). We also analyzed in a series of immunocytochemistry assays the effects of ZD1839 or C225 on the cellular

[8] Unpublished data.

distribution of total and activated ERK1/2. In both A431 and DiFi cells, treatment with these anti-EGF receptor compounds markedly decreased activated (phosphorylated) ERK1/2 staining and shifted total (phosphorylation-independent) ERK1/2 staining to a mainly cytoplasmic localization. These results suggested that treatment with ZD1839 or C225 prevents phosphorylation of ERK1/2 and results in the presence of nonphosphorylated ERK1/2 localized mainly in the cytoplasm.

We also studied whether an anti-EGF receptor compound could inhibit ERK1/2 activation in patients. This point was studied in a series of skin specimens, because skin is a well-characterized EGF receptor-dependent tissue (23–27). These studies showed that in patients treated with C225 ($n = 4$) there are markedly reduced levels of ERK1/2 activation in skin compared with skin from control patients ($n = 10$). Low levels of activated ERK1/2 in skin were seen in patients treated with C225 and radiation or C225 and cisplatin. In a patient that underwent sequential pre- and on-C225 skin biopsies, an evident inhibition of activated ERK1/2 was observed during therapy. This decrease in activated ERK1/2 was accompanied by a decrease in keratinocyte proliferation. This finding of a parallel effect of C225 treatment in patients on ERK1/2 activation and on proliferation in a human EGF receptor-dependent tissue further supports the notion of a biological effect of C225 on receptor downstream processes *in vivo*. In a similar line of evidence, a preliminary analysis in skin biopsies from patients treated with ZD1839 showed a significant decline in the expression of activated ERK1/2 in keratinocytes during therapy (38). We are extending these studies to a large set of skin biopsies from patients participating in ZD1839 Phase I trials to correlate the degree of ERK1/2 inhibition with clinically meaningful end points. The next challenge is to study activated ERK1/2 as a surrogate marker of EGF receptor inhibition in tumors from patients treated with anti-EGF receptor compounds. Such studies are currently ongoing based on the expression of activated ERK1/2 from our current series and the encouraging data obtained in nontumor skin biopsies post-C225 (current study) or post-ZD1839 therapy (38).

In summary, the data reported here show the presence of activated ERK1/2 in most primary head and neck squamous carcinomas. Elevated levels of activated ERK1/2 were associated with advanced lymph node metastasis and higher proliferation and were increased in relapses, suggesting a role for activated ERK1/2 in the malignant progression of these tumors. Our work also indicates that EGF receptor/TGF-α expression correlates with ERK1/2 activation *in vivo*. Furthermore, two different anti-EGF receptor treatments inhibited proliferation of EGF receptor-dependent model cell lines at concentrations that inhibited ERK1/2 activation. We also found that activation of ERK1/2 was lower in skin from patients treated with C225 compared with control skin, and this decrease in activated ERK1/2 was accompanied by a decrease in keratinocyte proliferation. As a result, we are incorporating assessment of ERK1/2 activation levels in pre- and post-therapy tumor biopsies from patients treated with the anti-EGF receptor tyrosine kinase inhibitor ZD1839 or with the MAb C225 to provide evidence for successful inhibition of EGF receptor function *in vivo* and to determine whether there is a correlation between levels of activated ERK1/2 and response to therapy.

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