[CANCER RESEARCH 59, 968–974, February 15, 1999]

Elevation of the Epidermal Growth Factor Receptor and Dependent Signaling in Human Papillomavirus-infected Laryngeal Papillomas[1]

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ABSTRACT

Laryngeal papillomas are benign tumors caused by human papillomaviruses types 6 and 11. This study addressed alterations in levels of signal transduction from the epidermal growth factor receptor (EGFR) in papillomas and cultured papilloma cells compared to normal tissue and cells. Mitogen-activated protein kinase (MAPK) was activated to a greater extent, phosphotyrosine was more abundant, and EGFR was overexpressed in laryngeal papillomas compared to normal laryngeal epithelium by Western blot analysis. The EGFR was 3 times more abundant in cultured papilloma cells than in normal laryngeal cells by Scatchard analysis and Western blot, without gene amplification or an increase in steady-state levels of mRNA. Following stimulation with EGF, a significant portion of the EGFR was recycled to the surface in papilloma cells, whereas in normal cells, it was not. Tyrosine kinase activity and activation of MAPK was more responsive to epidermal growth factor stimulation in papilloma cells than in uninfected primary laryngeal cells. PD153035, a specific inhibitor of the EGFR, and an EGFR-specific antibody that blocks ligand binding completely abrogated basal MAPK activation by endogenous ligands in laryngeal papilloma cells. These results demonstrated that infection of laryngeal epithelium by low-risk human papillomaviruses elevates the EGFR by posttranslational mechanisms, increasing its responsiveness to ligand-mediated activation. They also showed that MAPK activation in laryngeal papillomas depends upon ligand-mediated EGFR stimulation.

INTRODUCTION

Laryngeal papillomas are benign squamous epithelial tumors caused by low-risk HPV[5] type 6 or 11 (1, 2). They are characterized by a hyperplastic suprabasal epithelium surrounding cords of connective tissues (3). The expansion of the spinous layer most likely reflects a defect in the progression of cells through the normal program of differentiation. Papillomas express very little keratin 13 and essentially no filaggrin (4), which are normal differentiation markers of laryngeal epithelium. In cultured papilloma cells, this block to differentiation is relieved by removal of EGF from the medium (5).

The EGFR is a member of the erbB family of receptor tyrosine kinases. erbB receptors and EGFR in particular are often overexpressed in malignancies and derivative cell lines compared to normal epithelium. The EGFR responds to EGF and other cognate ligands by activating an intrinsic tyrosine kinase that generates a signaling complex by creating phosphotyrosine nucleation sites for SH2-containing proteins. However, ligands that bind EGFR at its external binding site are not the only mechanism through which the EGFR may become activated. Activation of G protein-coupled receptors may also stimulate phosphorylation of the EGFR (6). Activation of the EGFR results in a cascade of phosphorylations through the canonical ras-MAPK pathway affecting cell cycle regulation and differentiation (7). A key step in this process is the activation of ERK 1 and 2 (referred to as MAPK) by dual phosphorylation on threonine and tyrosine (8, 9).

Immunohistochemical analysis suggests that the EGFR is overexpressed in laryngeal papillomas (5). Moreover, removal of EGF from the medium of cultured papilloma cells provokes morphological and biochemical differentiation (5). However, the mechanism of this overexpression and its consequent effects on MAPK have not been addressed. Here, we quantitated overexpression of the EGFR and showed that it is a consequence of receptor recycling rather than elevated gene expression. We also demonstrated that the EGFR tyrosine kinase is hyperresponsive to EGF stimulation in cultured papilloma cells and that the basal activation of MAPK is completely dependent on EGFR kinase. The synthesis of the endogenous ligand TGF-α by these cells could account for basal levels of MAPK activity. The overexpression of the EGFR, enhanced phosphotyrosine content, and elevated activated MAPK were all consistent features of papilloma tissue, confirming that these differences in cultured cells reflected *in vivo* alterations in signaling.

MATERIALS AND METHODS

Tissues and Cell Culture. Epithelial explant cultures of normal human laryngeal cells or papilloma cells were established from biopsies in Ham's F12 with 10 μg/ml hydrocortisone and 10 ml/100 ml Fetal Clone II (Hyclone, Logan, UT) as described previously (4). The use of human tissues and cultured cells was approved by the Institutional Review Board at Long Island Jewish Medical Center. For experiments, cells were plated in serum-free KGM [supplemented keratinocyte basal medium (Clonetics, San Diego, CA) containing 0.5 μg/ml hydrocortisone, 2 μg/ml transferrin, 11 μg/ml bovine pituitary extract, and 1 nM all-*trans*-retinoic acid plus 1 ng/ml EGF and 5 μg/ml insulin]. Bovine pituitary extract was omitted from all signal transduction experiments.

Scatchard Analysis. Analysis was modified from Santos-Buch *et al.* (10). Briefly, cells were seeded at 50,000–80,000 per well in 96-well microculture dishes in KGM, cultured until they were 95% confluent, washed twice with ice-cold Krebs-Ringer binding buffer containing 10 mg/ml BSA, and then incubated for 1 h at 4°C with binding buffer containing 2 mg/ml BSA and increasing concentrations of [^{125}I]EGF (Amersham, Arlington Heights, IL), ranging from 0.2 to 5 ng/ml. To control for nonspecific binding, parallel wells were incubated with 500-fold excess unlabeled EGF as well as iodinated EGF. Cells were washed twice with ice-cold PBS, lysed with 100 μl of 1 g/100 ml SDS, and lysates were counted in a scintillation counter. Specific binding was determined by correcting total binding for nonspecific binding, and results were analyzed as per Scatchard (11). Results are the average of three experiments with each cell type, with each experiment done in duplicate.

Southern Blot Assessment of *EGFR* Gene Amplification. DNA from laryngeal papillomas and normal laryngeal tissues (9 μg each) was digested with *Eco*RI, electrophoresed through 8 mg/ml agarose gels, and transferred to nylon membranes. Blots were hybridized at 42°C with a 1000-bp *Eco*RI fragment of the human *EGFR* cDNA (pCO12-EGFR; Ref. 12), labeled by random priming with ^{32}P. To ensure that equal amounts of DNA were loaded in each lane, blots were probed simultaneously for the *proα2(I)* gene of human type I collagen (plasmid NJ3 3.55; Ref. 13), a single-copy gene. Blots were

Received 8/14/98; accepted 12/18/98.

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[1] This study was supported by National Institute on Deafness and Other Communication Disorders Grant P50-DC00203 and grants from the Morris S. and Florence H. Bender Foundation and the Irving and Helen Schneider Family Foundation.

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[5] The abbreviations used are: HPV, human papillomavirus; EGF, epidermal growth factor; EGFR, EGF receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; TGF-α, transforming growth factor α; KGM, keratinocyte growth medium; TBS, Tris-buffered saline.

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washed stringently in 0.1× SSC/1% SDS at 68°C. Autoradiograms were prepared at −70°C using intensifying screens and Kodak X-Omat AR film.

Slot Blot Analysis of *EGFR* mRNA. Normal and papilloma-derived laryngeal epithelial cells were seeded in KGM and mRNA isolated when the cells neared confluence (~4 days). mRNA (1 μg, on average) was slot-blotted onto membranes, hybridized at 42°C with a ^{32}P-labeled 1838-bp *Eco*RI fragment of the *EGFR* cDNA, washed stringently (T_m, −20), autoradiographed, and quantified by densitometry. The *EGFR* probe was removed by boiling for 20 min in 10 mM Tris (pH 8), 1 mM EDTA, and 10 mg/ml SDS, and the blot was reprobed with a ^{32}P-labeled 196-bp *Pst*I fragment of the *36B4* cDNA, a housekeeping gene encoding the acidic ribosomal phosphoprotein P0 (14). mRNA levels of *36B4* are constant within cells, with the protein levels regulated posttranscriptionally (15). The *EGFR* mRNA values were normalized to the *36B4* values to compare the different samples. The *EGFR* mRNA autoradiogram needed to be exposed ~20 times longer than the *36B4* autoradiogram.

Recycling of the EGFR. Cells were plated in 16-mm wells, cultured until they were 95% confluent, incubated overnight in KGM without EGF, and then incubated for 90 min at 37°C in EGF-free KGM containing 100 ng/ml cycloheximide to prevent new EGFR synthesis. Initial binding assays were performed as described for Scatchard analysis, using 4 ng/ml [^{125}I]EGF, to determine initial receptor levels. Parallel wells were then incubated with supplemented keratinocyte basal medium plus 100 ng/ml cycloheximide and 50 ng/ml EGF for 1 h and washed with PBS, and the noninternalized receptor was quantified in a subset of wells by [^{125}I]EGF binding. After internalization, a wash, and 5 h of incubation in EGF-free KGM containing 100 ng/ml cycloheximide, recycled receptor was measured on cells in remaining wells by [^{125}I]EGF binding. Internalization was defined as the reciprocal of the percentage of initial specific binding remaining at 1 h. Recycling of receptor was defined as the percentage of internalized receptor available for binding after 5 h incubation. Results are the means and SDs of two experiments with normal cells and three with papilloma cells, all from different patients. All experiments were performed in duplicate.

Immunofluorescent Detection of EGF. Normal and papilloma-derived laryngeal epithelial cells were grown on glass coverslips in KGM. To monitor the internalization of EGFRs into endocytic vesicles, cells were incubated for 24 h in medium lacking EGF, 100 ng/ml EGF was then added, and EGFR internalization was monitored at 0 time and 1 h later using indirect immunofluorescence. Cells were fixed with cold acetone for 5 min at −20°C, incubated with a 1:10 dilution of a mouse monoclonal antibody that reacts with the extracellular domain of the human EGFR (clone 2E9; Caltag Laboratories, San Francisco, CA), and then incubated with fluorescein-conjugated second antibody.

Western Blots. Cells were lysed by three cycles of dry ice freeze/ice thaw in lysis buffer [20 mM HEPES (pH 7.4), 137 mM NaCl, 2 mM EDTA, 10 μl/ml Triton X-100, 5 mg/ml deoxycholate, 1 mg/ml SDS, 0.1 ml/ml glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1.0 μM pepstatin, 50 μM NaF, 25 mM β-glycerophosphate, and 1 mM $NaVO_4$]. Nuclei remaining on the plate were lysed with Laemmli sample buffer (16) to yield triton-insoluble extracts. Biopsy tissues were extracted by thawing snap-frozen specimens and sonicating in lysis buffer. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL), and 20 μg of total protein were loaded per lane on polyacrylamide gels containing 0.1 g/ml SDS. Gels were electroblotted onto polyvinylidene difluoride membranes, and blots were stained with fast green (17) to confirm equivalence in loading and transfer. Nonspecific binding to the filters was blocked by preincubating in TBS buffer [20 mM Tris (pH 7.4) and 9 mg/ml NaCl] plus 5% BSA, and the filters were incubated with primary antibodies for 1 h in TBS plus 0.05% Tween 20 and 1% BSA with shaking at room temperature, washed, and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Following extensive washing in TBS plus 0.05% Tween 20, reactive antigens were detected using chemiluminescence. Monoclonal antiphosphotyrosine, anti-EGFR (LA1), and anti-ERK-1 (all from Upstate Biotechnology Inc., Lake Placid, NY) were used at 1:1000 dilution. Polyclonal rabbit antibodies anti-EGFR (Calbiochem, San Diego, CA) and ERK-1 NT (Transduction Labs, Lexington, KY) were used at 1 μg/ml. Anti-activated MAPK (Promega, Madison, WI) was used at 1:10,000 dilution. Anti-TGF-α (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 200 ng/ml.

Inhibition by PD153035 and LA1. Confluent monolayer cultures were incubated in KGM without EGF for 3 days and then incubated in the same medium containing either 1 μM PD153035 (Tocris Cookson, St. Louis, MO) for 30 min or 5 μg/ml LA1 (Upstate Biotechnologies Inc.) for 2 h. Control cultures were incubated with an irrelevant monoclonal antibody that recognizes TGF-α (Santa Cruz) but does not inhibit binding of TGF-α to the EGFR, at 5 μg/ml. Cultures were lysed as above for Western blot analysis.

EGFR Thresholds for Tyrosine Phosphorylation and MAPK Activation. Cells were cultured in KGM until they were subconfluent; incubated in KGM without EGF for 3 days; pulsed for 10 min with 0, 0.1, 1, 10, or 100 ng/ml EGF; and analyzed by Western blot as described above.

RESULTS

EGFR Is Overexpressed in Laryngeal Papillomas. Our earlier study on EGFR overexpression in papilloma cells was based on immunohistochemical staining of organotypic raft cultures, in which both basal and stratifying layers of epithelial cells are present (5). In this study, Western blots of laryngeal tissue demonstrated that a consistent feature of papillomas is the extreme abundance of phosphotyrosine-containing proteins (Fig. 1). Of a total of nine papilloma extracts probed for phosphotyrosine (data not shown), all had elevated levels when compared to extracts of normal laryngeal tissues. To determine whether the levels of phosphotyrosine found in papillomas correlated with changes in the abundance of the EGFR and the activation of MAPK, the Western blot was sequentially reprobed for the EGFR, for activated MAPK, and for total ERK-1 (Fig. 1). The responses indicated that, for a fixed level of ERK-1, the EGFR was more highly expressed in papillomas, phosphotyrosine content was radically elevated, and MAPK was activated to a greater extent than in normal laryngeal tissue. The apparent difference in molecular weight of the EGFR in lysates of laryngeal papillomas (Fig. 1, *Lanes 3* and *4*) may reflect variation in phosphorylation or posttranslational modification of the EGFR among tissues from different patients.

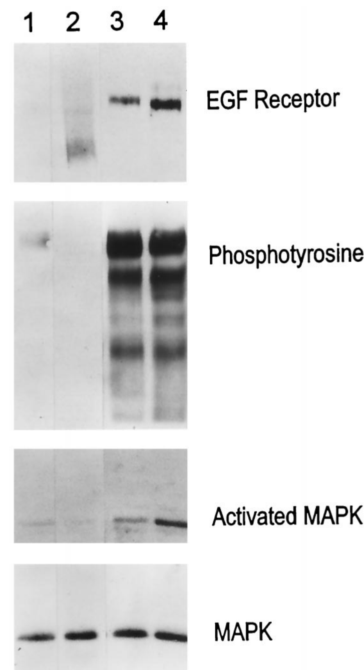

Fig. 1. Western blot of extracts from normal and papilloma tissues. Snap-frozen biopsies of normal (*Lanes 1–2*) and papilloma (*Lanes 3–4*) tissues were extracted and analyzed by western blot with antibodies directed against the EGFR, phosphorylated tyrosine, activated MAPK, and total ERK-1 MAPK. Equivalent protein loading and transfer was assured by staining filters with fast green (data not shown).

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EGFR Is Three Times More Abundant in Papilloma Cells Than in Normal Cells, Due to Recycling. The overexpression of the EGFR was also seen in monolayer cultures of papilloma cells in low-calcium, serum-free medium, analyzed by Western blots of Triton X-100-soluble cell extracts (Fig. 2). Similar to the results found in tissue, the levels of the EGFR in four independent papilloma cultures were consistently higher than those found in uninfected laryngeal cells. We then asked whether the overexpression of the EGFR on papilloma cells could be confirmed with EGF binding studies, using monolayer cultures of normal and papilloma cells. This approach allowed quantitative determination of both receptor number and ligand affinity on the proliferative basal cell component. Scatchard analysis of the binding studies (Fig. 3) confirmed that the papilloma cells expressed 3 times more high-affinity receptors on their surface than the cultured normal laryngeal keratinocytes but that the affinity of the receptors for EGF was the same (K_d, 1 nM).

To determine the mechanism of EGFR elevation, we found, by Southern blot analysis, that the gene was neither amplified in cultured laryngeal papilloma cells (data not shown) nor overexpressed at the mRNA level when analyzed by RNA slot blot (Table 1). Another possible explanation for EGFR overexpression was a defect in internalization or degradation of the EGFR in response to ligand binding, similar to that reported for HPV 16 *E5*-transfected keratinocytes (18). To test for a block in initial internalization, normal, and papilloma cells were starved of EGF to maximize surface receptor number. EGF was then replaced and internalization of receptor into endocytic vesicles was monitored by indirect immunofluorescence (Fig. 4). EGFRs

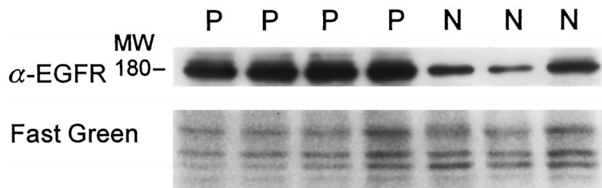

Fig. 2. EGFR levels in normal and papilloma cells. Cells were cultured in KGM and lysed, and Triton-soluble extracts were analyzed by Western blot for EGFR. *Lanes P*, papilloma cells; *Lanes N*, normal laryngeal epithelial cells. Fast green stain of filter is shown to confirm equal protein in each lane.

Table 1 *EGFR RNA levels in cultured laryngeal keratinocytes*[a]

	Relative intensity	
Experiment no.	Normal cells	Papilloma cells
1	1.7	2.0
2	2.5	0.6
3	1.2	0.7
4	0.4	0.7
5		0.8
6		0.6
7		0.9
Average	1.5	0.9

[a] RNA from cells was extracted and analyzed by slot blot hybridization with a ^{32}P-labeled *EGFR* cDNA probe. Intensity (relative densitometric units) was normalized to the *36B4* housekeeping gene. All samples were analyzed on the same blot. The *EGFR* autoradiogram was exposed for 22 h, whereas the *36B4* autoradiogram was exposed for 1 h.

were internalized into punctate intracytoplasmic organelles in both normal and papilloma-derived cells within 1 h of addition of ligand.

Although internalization appeared normal, subtle defects in internalization would not be delectable using this approach. To more precisely determine the extent of internalization of the EGFR, and possible recycling of receptors to the cell surface, we used binding studies with [^{125}I]EGF at varying times after exposure to excess unlabeled EGF (Table 2). These experiments were performed in the presence of cycloheximide to prevent *de novo* receptor synthesis. They confirmed that both types of cells had essentially internalized all receptor 1 h after the EGF pulse but that HPV 11-infected papilloma cells recycled at least 20% of the EGFR within 5 h following ligand binding. Normal laryngeal cells recycled minimal EGFR. From these studies, we concluded that receptor recycling accounted for the increased number of EGFR on papilloma cells.

Papilloma Cells Have a Lower Threshold for EGF-induced Activation of MAPK. The basal level of phosphotyrosine in either cell type in culture was low but detectable. A 10-min exposure to 10 ng/ml EGF-stimulated phosphorylation of tyrosine on proteins at M_r 180,000, 65,000, 55,000, and 42,000–44,000 (Fig. 5). The response of primary uninfected laryngeal cells to 10 ng/ml EGF varied somewhat in intensity among different cultures, but the patterns were consistent. Evidence of EGF response in these cells was most apparent with phosphorylation of the M_r 180,000 protein and barely detectable

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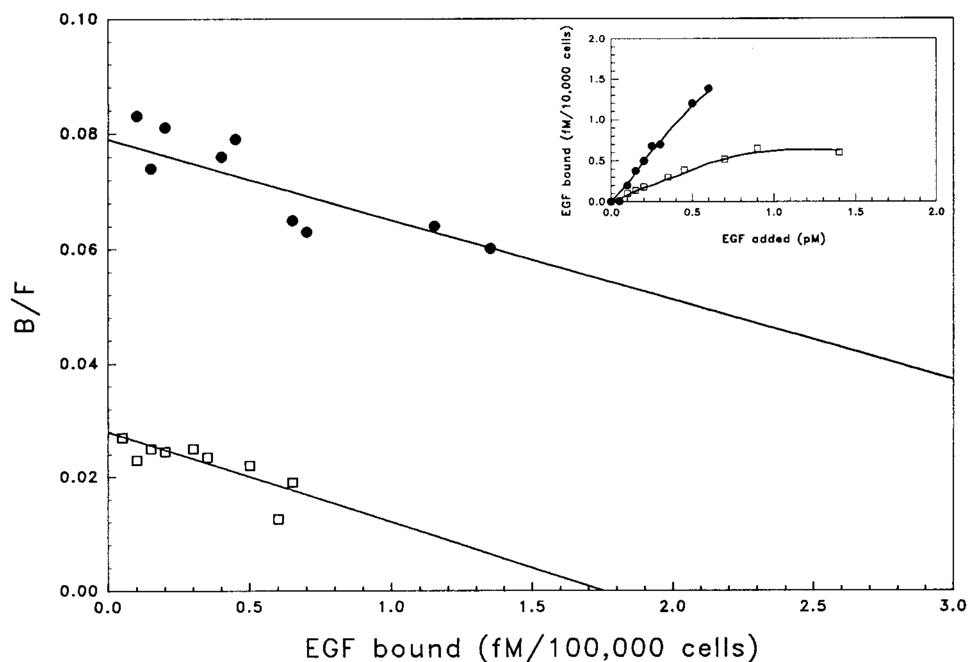

Fig. 3. Scatchard analysis of EGFR number and affinity on normal and papilloma cells. Cells cultured in KGM were incubated with [^{125}I]EGF concentrations ranging from 0.2 to 5 ng/ml. To control for nonspecific binding, parallel wells were incubated with 500-fold excess unlabeled EGF as well as labeled EGF. Specific binding (shown) was determined by correcting total binding for nonspecific binding. K_d and receptor number were calculated as per Scatchard (11). *Data points*, averages of three experiments with each cell type, with each experiment performed in duplicate.

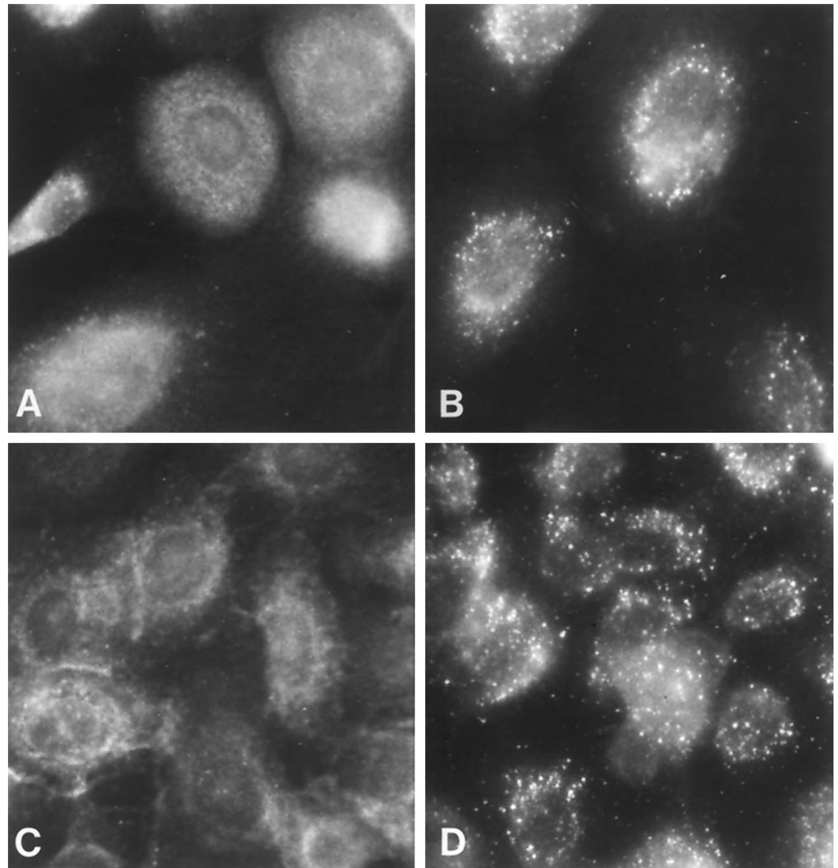

Fig. 4. Internalization of the EGFR following ligand binding. Normal (*A* and *B*) and papilloma (*C* and *D*) cells were incubated in KGM without EGF for 24 h, and 100 ng/ml EGF was added to the medium. EGFR distribution at 0 time (*A* and *C*) and after 1 h incubation (*B* and *D*) was determined by indirect immunofluorescence, using an antibody that recognizes the extracellular domain of the EGFR.

Table 2 *Recycling of EGFR*[a]

Cell type	% receptor internalized	% receptor recycled
Normal	93.0 ± 4.2	6.0 ± 0.05
Papilloma	93.3 ± 9.0	21.2 ± 2.0

[a] Cells were cultured overnight in KGM lacking EGF and preincubated with 100 ng/ml cycloheximide for 90 minutes to prevent further protein synthesis, and [^{125}I]EGF binding to the cell surface measured at 0 time and at 1 h (used to calculate percentage internalized receptor) and 5 h (used to calculate percentage recycled receptor) after a 1-h pulse with excess (50 ng/ml) EGF. Cycloheximide was present throughout the incubation time.

phosphorylation of the M_r 65,000 protein. There was little or no change in the tyrosine phosphorylation of the M_r 42,000 protein in this culture. In contrast, the papilloma cells showed strong phosphorylation of the M_r 180,000, 65,000, and 55,000 proteins, and an increase in phosphorylation of the M_r 42,000 protein with 10 ng/ml EGF. They showed clearly detectable phosphorylation of the M_r 180,000 band at 1 ng/ml EGF, and phosphorylation of the M_r 65,000 band at 0.1 ng/ml.

The highest molecular weight band was not due to autophosphorylation of the EGFR (see "Discussion"). This protein may be erbB-2, as has been seen in transfection experiments with HPV 16 E5 (19). The bands at M_r 44,000–42,000 reflect tyrosine phosphorylation of ERK-1 and -2 as a result of activation of the canonical ras/MAPK pathway. However, detection of phosphotyrosine may not accurately reflect the relative levels of activated MAPK because this requires dual phosphorylation of both threonine and tyrosine residues.

The papilloma tissues (see Fig. 1) had shown an increase in both phosphotyrosine and activated MAPK when compared to normal tissue, suggesting a possible difference in threshold for MAPK activation by the *in vivo* endogenous ligands for the EGFR. Moreover, the increased tyrosine phosphorylation of the M_r 42,000 MAPK in papilloma cells, shown in Fig. 5, supported this possibility. We, therefore, asked what concentration of EGF was required to activate MAPK in normal and papilloma cells (Fig. 6). Papilloma and normal cells were starved of exogenous growth factors for 3 days and then pulsed with increasing amounts of EGF. At 0.1 and 1 ng/ml EGF, the normal cells showed no increase in phosphorylation of the M_r 180,000 erbB family of proteins. In contrast, tyrosine phosphorylation was detectable in papilloma cells with 0.1 and 1 ng/ml EGF. Papilloma and normal cells both exhibited tyrosine phosphorylation with 10 ng/ml EGF.

Normal and papilloma cells showed marked differences in EGF threshold for activation of the M_r 42,000 Triton-insoluble ERK-1 MAPK above basal levels with minimal activation of the M_r 42,000 ERK-2. To facilitate comparison, film exposure times for the blots of

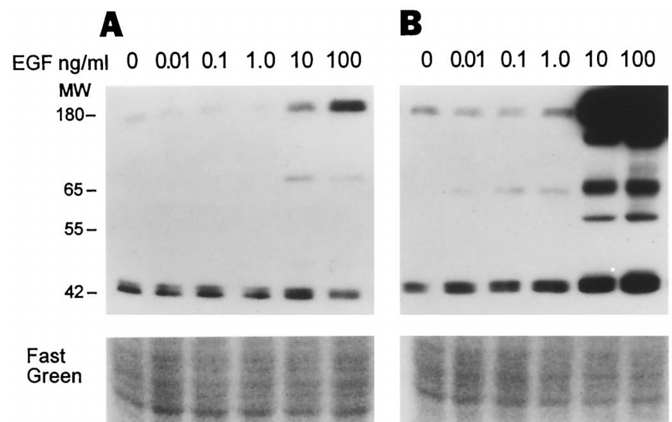

Fig. 5. Phosphotyrosine levels after pulsing normal and papilloma cells with EGF. Cells were cultured in KGM without EGF and pulsed for 10 min with 10 pg/ml to 100 ng/ml EGF, and Triton-soluble extracts were analyzed by Western blot. Fast green stains of each filter are shown to illustrate equal protein levels. *A*, normal laryngeal cells. *B*, papilloma cells. *MW*, approximate molecular masses in kilodaltons.

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papilloma and normal cultures were adjusted to give the same intensity for basal MAPK activation. Papilloma cells activated the M_r 42,000 ERK-1 to nearly full levels when pulsed with 0.1 ng/ml EGF, whereas normal cells did not respond at all to this concentration. Even at 10 ng/ml, the normal cells did not achieve the relative increase in active MAPK seen with papilloma cells pulsed with 0.1 ng/ml EGF.

MAPK Activation Depends on the EGFR Kinase. A basal level of MAPK activation was seen in both cell types when starved of EGF for 3 days. We, therefore, asked whether the basal levels were a result of EGFR activation. To address this question, we used PD153035, a specific inhibitor of the EGFR tyrosine kinase (20). This drug completely eliminated the basal level of activated MAPK in papilloma cells (Fig. 7). This was consistent with a dependence of basal activation of MAPK on the EGFR tyrosine kinase.

The EGFR may be activated as a result of binding ligand, through heterologous activation such as heterodimerization with other members of the erbB family, or through tyrosine phosphorylation as a secondary effect of stimulation by ligands of G protein-coupled receptors (6). To determine whether the EGFR-dependent MAPK activation required direct binding of an external ligand, we incubated normal and papilloma cells with an antibody, LA1, that competitively blocks ligand binding (21). LA1 completely abrogated MAPK activation (Fig. 7), indicating that basal MAPK activation depends upon ligand occupancy of the EGFR.

Because LA1 blocked MAPK activation in both cell types, we asked whether ligands of the EGFR were produced by the cells. Western blots of Triton X-100-soluble extracts detected bands at the molecular weight of the TGF-α precursor (M_r ~8,000–12,000, depending on extent of posttranslational modification and partial cleavage) in both papilloma and normal tissues and cultured cells (Fig. 8). This was consistent with an autocrine mechanism for basal MAPK activation. There were interesting differences in the expression of TGF-α between biopsy lysates and cultured cells. Although there was obvious quantitative variation on this blot, these were due, at least in part, to differences in protein loaded on the gel. We have presented these data as evidence of the presence of an endogenous EGFR ligand that could account for the LA1-inhibitable activity. They were not intended to be evidence of quantitative expression differences among these sample types. The variation in molecular weights of TGF-α between biopsy lysates and cultured cells was, however, consistent enough to advance the speculation that growth of cells in culture affects the synthesis or processing of this protein. TGF-α undergoes multiple modifications during its maturation, including glycosylation and proteolysis. It is possible that the reduced molecular weights of the TGF-α forms seen in cultured cells are the result of a failure to glycosylate the peptide or a facile cleavage not catalyzed in intact tissues.

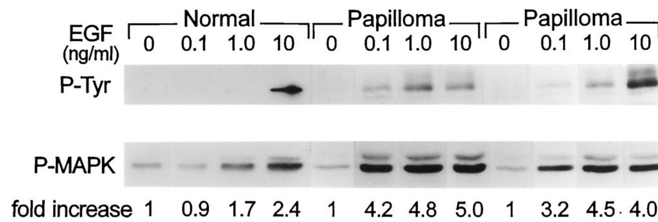

Fig. 6. Thresholds for activation of MAPK by EGF. Normal cells and two different cultures of papilloma cells were maintained in KGM with no growth factors for 3 days and then pulsed for 10 min with medium containing varying concentrations of EGF. Cell extracts were analyzed by Western blot using chemiluminescent detection, and films were analyzed by densitometry. The normal and papilloma cells were analyzed in separate experiments. To facilitate comparison of relative activation, the film exposures were adjusted to give equal intensity of MAPK activation without EGF. *P-Tyr*, Triton-soluble extracts probed with antibody to phosphotyrosine-containing proteins; M_r 180,000 band shown. *P-MAPK*, Triton-insoluble extracts probed with antibody to activated MAPK. *fold increase*, activation of MAPK relative to the control cells without EGF. Fast green staining of filters confirmed equal loading (data not shown).

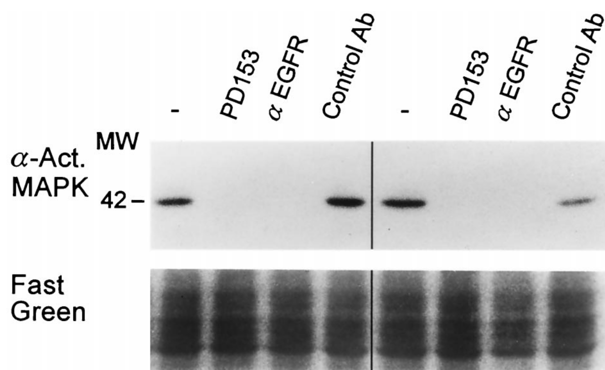

Fig. 7. Effects of EGFR inhibitors on MAPK activation in papilloma cells. Cells were cultured in KGM without EGF for 3 days and treated with EGFR inhibitors, and MAPK activation in Triton-soluble extracts was determined by Western blot. *PD153*, 1.0 μM PD153035 for 30 min; *α EGFR*, 5 μg/ml antibody LA1 incubated with cells for 2 h; *Control Ab*, 5 μg/ml irrelevant monoclonal antibody, used as a control for LA1. Fast green stain of filter confirms equal protein in each lane.

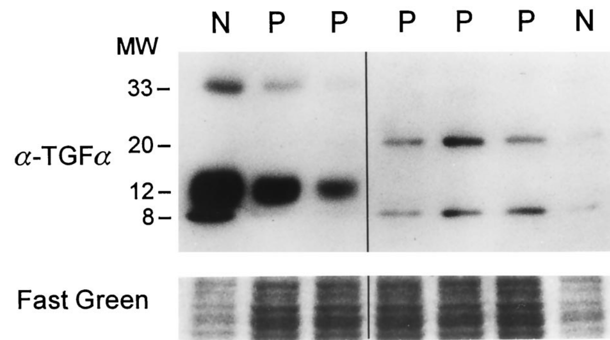

Fig. 8. Expression of TGF-α by normal and papilloma cells in culture. Cells were cultured in serum-free KGM in the absence of EGF, lysed, and analyzed by Western blot for the presence of TGF-α and its precursor forms. *MW*, approximate molecular masses in kilodaltons. Fast green stains indicate variability in protein in these lanes, precluding accurate analysis of relative amounts of TGF-α.

DISCUSSION

Cultured laryngeal papilloma cells are a model system for the study of the effects of epithelial infection by low risk HPV. In contrast to immortalized cell lines, these primary cells closely reflect the biology of *in vivo* papillomas. In this report, we have described the perturbations of EGFR signaling that results from HPV infection of the laryngeal mucosa, expanded our understanding of EGFR signaling by demonstrating a dependency of MAPK signaling in papillomas on both the EGFR tyrosine kinase and EGFR receptor occupancy and demonstrated the presence of TGF-α as an endogenous product of laryngeal epithelium. Our cell culture experiments explain the elevation of the EGFR and the increased levels of phosphotyrosine and MAPK activation that are apparent in papillomas.

The overexpression of the EGFR was due to posttranslational recycling of the EGFR and not to an enhancement of transcription. This response to HPV 6/11 infection is similar to the previously described effect of HPV 16 E5 protein on EGFR recycling in foreskin epithelial cells (17). Cells transfected with HPV 16 *E5* and HPV 6 *E5a* respond to EGF in clonogenic assays for transformation, and HPV 6 E5a has been shown to associate with both the EGFR and erbB-2 (22, 23). The low-risk HPV genomes in laryngeal papillomas exist as

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unintegrated episomes, and transcripts arising from the viral promoter region contain *E5a*-encoding sequences (24). Thus, our data are consistent with elevation of the EGFR via an HPV 6- or HPV 11 E5a-mediated effect.

The status of epithelial differentiation may affect the EGFR, independent of HPV infection. Therefore, because HPV infection affects the state of differentiation (4), the function and abundance of the EGFR could be a secondary effect of infection rather than a direct result of one or more HPV gene products. EGFR expression diminishes in suprabasal layers of normal human epithelium. Inhibition of the completion of terminal differentiation by HPV infection could, therefore, contribute to an overexpression of the EGFR. However, cells cultured from papillomas are necessarily from the proliferative compartment of the laryngeal epithelium. We would therefore expect that cultured cells would retain the same level of the EGFR found in uninfected cells, if the state of differentiation were the only factor in determining receptor expression. This is not the case. Instead, we find enhanced expression of the EGFR in both the papillomas and in their cultured cells. This is more consistent with a direct effect of HPV gene expression on EGFR levels than with a change in EGFR levels as a secondary result of infection altering differentiation.

The EGFR kinase was hyperresponsive to EGF in papilloma cells. This responsiveness was reflected in the abundance of phosphotyrosine incorporated into proteins at M_r 180,000, 65,000, 55,000, and 42,000–44,000. However, the largest of these proteins was not the EGFR because, in the 10-min treatment we used, the EGFR was completely lost from the Triton-soluble fraction (data not shown). Similarly, Daub *et al.* (6) reported that a 5-min treatment of COS-7 cells with 30 ng/ml EGF resulted in the disappearance of Triton-soluble EGFR but an increase in phosphotyrosine at M_r 170,000. We have ascribed this phosphorylation target to erbB-2 because it has been shown that erbB-2 is a target for hyperresponsive EGFR in HPV 16 *E5* transfection experiments (19). erbB-2, erbB-3, and erbB-4 were not overexpressed in laryngeal papillomas, although all were present (data not shown). This was consistent with specific enhancement of EGFR abundance due to receptor recycling following ligand mediated internalization because none of remaining erbB family members undergo ligand-mediated internalization and, therefore, would not increase by that recycling mechanism (25). The phosphorylated bands at M_r 55,000 and 65,000 are presently unidentified. However, EGFR targets at these approximate molecular weights have been ascribed to isoforms of shc. The band at M_r 65,000 was highly sensitive to EGF-stimulated phosphorylation in papilloma cells, acquiring phosphotyrosine at 10 pg/ml EGF. Hyperphosphorylation at low concentrations of EGF was less evident in the bands that we ascribed to ERK-1 and -2, because these were already highly phosphorylated on tyrosine in both normal and papilloma cells. It is important not to construe this as evidence of a comparable enhancement of basal activated (as opposed to tyrosine-phosphorylated) MAPK in papilloma cells compared to normal cells because activation requires both threonine and tyrosine phosphorylation. Our data suggests that this was not the case in EGF-starved cells in monolayer cultures (data not shown).

The heightened response of papilloma cells to EGF could be a simple result of mass action because, for a given level of ligand, a greater number of receptors would yield a greater activation of tyrosine kinases. However, the lack of any observable change in phosphorylation at M_r 180,000 in normal cells treated with low concentrations of EGF argues for a qualitative difference between infected and uninfected cells. This difference may reflect the observation that HPV E5 proteins not only inhibit receptor degradation by binding the vacuolar (H+)-ATPase, but also bind the EGFR and erbB-2 (23). The nature of the direct effect of HPV 11 E5a on EGFR-dependent signaling is currently under study in our laboratory.

Sustained MAPK activation has been described as necessary for onset of differentiation in PC12 cells following transfection of oncogenes or treatment with nerve growth factor (26, 27). However, by itself, sustained activation of MAPK is insufficient for PC12 differentiation (28). In contrast, MAPK activation inhibits murine adipocyte differentiation, indicating that the effect of MAPK activation on differentiation is cell type specific (29). Our previous data indicated that a prolonged withdrawal of EGF from cell culture media induced morphological and biochemical markers of differentiation in papilloma cells growing on organotypic rafts (5). Thus, MAPK activation in papilloma cells does not provoke morphological differentiation and may be a mechanism to inhibit the differentiation of these cells. This hypothesis is in agreement with the observation of elevated MAPK in papilloma tissues that are characterized by a block in differentiation.

Here, we demonstrated a basal activation of MAPK in monolayer cultures of papilloma cells following EGF withdrawal, dependent on both tyrosine kinase activity and ligand occupancy of the EGFR. Thus, it might be expected that removal of EGF would not permit differentiation of either cell type. However, our previous studies (5) showed that expression of the EGFR was limited to basal cells in the normal cell raft cultures, although it extended throughout most of the spinous layer in the papilloma cell raft cultures. Thus, the increased sensitivity of suprabasal papilloma cells to MAPK activation by EGF could explain the ability of normal cells on raft cultures to differentiate in the presence of EGF, although papilloma cells were blocked.

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