Laryngeal Papilloma Cells Have High Levels of Epidermal Growth Factor Receptor and Respond to Epidermal Growth Factor by a Decrease in Epithelial Differentiation

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ABSTRACT

Laryngeal papillomas are benign epithelial tumors caused by human papillomaviruses. These tumors are characterized by hyperplasia of the spinous layer and abnormal differentiation. Many tumor cell lines overexpress the epidermal growth factor (EGF) receptor on their surface, and EGF regulates normal cell growth. We have asked about the relationship of the EGF receptor and EGF response in laryngeal papilloma cells. Papilloma cells showed markedly greater immunohistochemical staining for the EGF receptor, compared to uninfected cells. Both cell types showed a 2-3-fold increase in nuclei incorporating bromodeoxyuridine when EGF was present. Removal of EGF from papilloma cells cultured on collagen rafts permitted normal stratification and differentiation, as determined by synthesis of keratin 13. Inclusion of EGF induced abnormal differentiation with minimal expression of keratin 13. Uninfected laryngeal cells cultured on rafts in the presence of EGF synthesized keratin 13 in all suprabasal cells. EGF reduced both human papillomavirus RNA levels in the papilloma cells and expression of a reporter gene linked to the human papillomavirus 11 enhancers and E6 promoter in uninfected cells. These results suggest that the phenotype of papillomas is induced, in part, by EGF binding to the abundant EGF receptors.

INTRODUCTION

Human laryngeal papillomas are benign squamous epithelial tumors induced by HPV\(^3\) types 6 and 11 (1). Approximately 30% convert to squamous cell carcinomas after therapeutic X-irradiation (2). Spontaneous conversion is less likely but does occur (3). The papillomas might therefore be considered premalignant lesions. As such, they can serve as models for early neoplastic changes. The papillomas are characterized by a hyperplastic spinous epithelial layer, with a basal layer that may be normal or hyperplastic and a defect in differentiation that results in the reduction or absence of differentiation-specific keratin 13 (4). When normal epithelial cells are cultured at the air-liquid interface on a raft of collagen containing fibroblasts (raft cultures), they complete the differentiation process (5). In contrast, the papilloma cells continue to show the reduction in K13 expression (6).

The growth of epithelial cells is regulated in part by the binding of EGF to the EGF receptor. EGF stimulates the proliferation of normal epithelial cells in vivo (7, 8) and in vitro (9, 10). In vitro, there is an inverse relationship between EGF response and differentiation in a number of different cell types (11, 12). Effects of EGF can be altered when other regulatory factors such as retinoids or transforming growth factors are present (13). In fact, transforming growth factor \(\alpha\) binds to the EGFR and induces many of the same events as EGF (14).

The EGFR is a member of the tyrosine kinase receptor family (for a review see Ref. 15). The receptor is often overexpressed in tumors and tumor cell lines, sometimes in conjunction with gene amplification (16-18). Overexpression of the cloned human EGFR cDNA in NIH 3T3 cells is sufficient to induce EGF-dependent transformation (19).

Previous studies have reported the overexpression of EGFR in some benign papillomavirus-induced lesions (20, 21). Some HPV-16 transformed cell lines and cervical cancer cells express high numbers of EGFR, as do some bovine papillomavirus transformants (22, 23). However, Doeberitz et al. (24) found that altering HPV-18 expression in a cervical cell line with glucocorticoids caused changes in growth properties that correlated with viral E6/E7 protein levels but not with EGFR expression. Pim et al. (25) recently reported that HPV-16 E6 transfectants show an exaggerated growth response to EGF but do not overexpress the EGFR. Therefore, whether regulation and/or overexpression of the EGFR is a dominant and central mechanism of HPV-mediated growth control is still not resolved.

In this study, we have asked whether cultured cells derived from laryngeal papillomas express high levels of EGFR on their surface. We have also asked what effect(s) EGF has on the growth and differentiation of these cells and on the expression of their viral RNA.

MATERIALS AND METHODS

Tissues and Cell Culture. Primary human laryngeal cells were cultured as previously described from biopsy fragments of normal and papillomatous tissues (26). Use of this tissue has the approval of the Long Island Jewish Medical Center Human Investigations Committee and is in accordance with the regulations of the Department of Health and Human Services. Informed consent was obtained for the biopsies at the time of scheduled surgery. No surgery was performed simply to obtain biopsies.

All experiments were carried out with first-passage cells grown in serum-free keratinocyte basal medium (Clonetics) supplemented with 2 ng/ml transferrin, 0.5 \(\mu\)g/ml hydrocortisone, 21 ng/ml pituitary extract, 5 \(\mu\)g/ml insulin, 1 \(\mu\)g/ml retinoic acid, and the desired concentration of calcium and EGF. The standard concentration of calcium in this medium is 0.1-0.15 mm, and EGF is 1 ng/ml. Experiments were conducted three times with each cell type except where noted, using cells derived from different biopsies from different patients.

For organotypic studies, cells were grown on collagen rafts at the air-liquid interface as previously described (5). They were plated in KGM containing 0.7 \(\mu\)mol/L high calcium (for normal cells) or 1.0 \(\mu\)mol/L calcium (for papilloma cells) at a density of 1 \(\times\) 10^5 cells/cm^2. These conditions were previously determined to be optimal for stratification. When confluent, cultures were lifted to the air-liquid interface, fed with KGM lacking EGF for 48 h, and then fed with medium containing 0, 1, or 50 ng/ml EGF for an additional 72 h as described by Yasumoto (27). RNA was then isolated, or cells were embedded for frozen sections.

For growth studies, cells from a single biopsy were plated on four glass coverslips at 5 \(\times\) 10^5 cells/cm^2 in standard KGM and cultured until subconfluent. They were then fed medium containing either 0.15 \(\mu\)mol (low) or 1.0 \(\mu\)mol (high) calcium and either no EGF or 10 ng/ml EGF for 96 h. Three \(\mu\)mol bromodeoxyuridine/ml were added for the last 24 h. Incorporation into proliferating cells was determined using an antibody to bromodeoxyuridine and counting total stained nuclei as previously described (28). Briefly, 15 fields were counted per coverslip, with approximately 150 cells/field. Data are counted total stained nuclei as previously described (28). Briefly, 15 fields were counted per coverslip, with approximately 150 cells/field.
expressed as means and SDs of the percentage labeled nuclei. Experiments were carried out with two cultures of normal cells and three cultures of papilloma cells.

For transient expression studies, normal cells were plated on marked 10-mm glass coverslips in standard KGM at a density of $5 \times 10^4$/cm$^2$. When confluent, the cells were fed with KGM containing no EGF for 48 h and then fed with KGM containing 0.1, or 50 ng/ml EGF for 72 h and used for assay.

**Detection of EGF and K13.** Epithelialized collagen rafts were embedded in OCT and snap-frozen in liquid nitrogen. Four-μm sections were air dried before fixation and incubation with the desired antibody (see below). Antibodies were detected with a second antibody–alkaline phosphatase–biotinylated streapavidin detection system (StrAviGen; Biogenex, Inc.) To detect EGF, sections were fixed in cold acetone. Mouse monoclonal anti-EGFR (Caltag Laboratories, San Francisco, CA) was used at a dilution of 1:90. For K13 detection, sections were fixed in cold methanol. Mouse monoclonal antibody AE8 (29), generously provided by Dr. T-T. Sun, was used as an undiluted culture supernatant. To control for staining variability, multiple specimens that had been stored at −80°C were stained in the same experiment. All specimens from a single culture were stained together, and normal and papilloma specimens were stained together.

**HPV RNA Analysis.** Epithelial layers were peeled from collagen rafts after submersion in 50 units/ml dispase (Collaborative Research) and trypsinized. Cytoplasmic RNA was isolated as described (30) and treated with 0.004 units DNase/μl to remove any contaminating DNA. The RNA samples were slot-blotted to Gene Screen Plus (NEN Research Products) as recommended by the vendor. Filters were hybridized with $^{32}$P-labeled HPV 6 or 11 DNA excised from cloned DNA with BamHI, washed, and autoradiographed as previously described (31). If the HPV type in the cells was unknown, a mixed HPV 6/11 probe was used. Standards consisted of cloned denatured HPV 6 and 11 DNA (generously provided by Dr. Lutz Gissmann) and human tonsil DNA. All hybridizations and washes were carried out under stringent conditions (7°C).

**RESULTS**

**Expression of the EGF Receptor.** We first compared the effects of EGF on expression of the EGFR in both normal and papilloma cells. Raft cultures were used for these studies because they permit normal stratification and differentiation of laryngeal keratinocytes. When uninfected cells were cultured in KGM with 1 ng/ml EGF, there was barely detectable EGFR staining in the basal layer and no staining of suprabasal layers (Fig. 1A). When EGF was removed from the cultures for 5 days, there was an increase in staining that also extended throughout most of the epithelium (Fig. 1B). Refeeding with EGF after 48 h of depletion eliminated staining of the EGFR (Fig. 1, C–D). This pattern of response was expected, since binding of EGF to the EGFR results in internalization and degradation of the receptor in normal epithelial cells. Maximal levels of receptor would be seen in the absence of ligand.

In contrast, papilloma cells cultured continuously in 1 ng/ml EGF displayed very heavy staining of the basal layer and significant staining throughout the lower half of the epithelium (Fig. 1E). Depletion of EGF reduced staining of the receptor (Fig. 1F). This is in contrast to expected results and in contrast to the uninfected cells. Refeeding with 1 ng/ml EGF after 48 h of depletion (Fig. 1G) gave a pattern very similar to that seen with continuous exposure to EGF. Even with the addition of 50 ng/ml EGF (Fig. 1H), the staining of basal receptors was not abolished. These staining patterns show that the steady-state levels of EGFR are markedly higher in laryngeal papilloma cells and suggest that regulation of the receptor is different. When uninfected and papilloma cells were cultured on submerged glass coverslips in standard KGM there was a similar difference in EGFR staining intensity between the two cell types (data not shown).

**Effects of EGF on Cell Growth and Differentiation.** We examined the effects of EGF on the growth of the uninfected and papillo-
Table 1 Effects of EGF and calcium on the proliferation of normal laryngeal cells and papilloma cells

<table>
<thead>
<tr>
<th>Calcium</th>
<th>Normal cells</th>
<th>Papilloma cells</th>
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<tr>
<td></td>
<td>(-) EGF</td>
<td>(+) EGF</td>
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<tr>
<td>Low</td>
<td>16.5 ± 7.5</td>
<td>33.5 ± 3.5</td>
</tr>
<tr>
<td>High</td>
<td>19.0 ± 2.8</td>
<td>52.0 ± 0</td>
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We then evaluated the effect of EGF on expression of keratin 13, a marker for normal differentiation in laryngeal cells. Uninfected cells express K13 in all suprabasal cells when grown on raft cultures with 1 ng/ml EGF in the medium (5). The response of papilloma cells was quite different (Fig. 2). Under standard culture conditions, with 1 ng/ml EGF present (Fig. 2A), we saw the very limited K13 staining that has previously been described both in vivo and in vitro (4, 6). When EGF was removed from the medium for 5 days (Fig. 2B) there was extensive suprabasal staining. This was comparable to the K13 staining pattern in uninfected cells in vivo and in vitro (4, 5). Replacement of EGF at 1 ng/ml (Fig. 2C) or 50 ng/ml (Fig. 2D) for 72 h resulted in intermediate levels of staining, with weakly positive cells restricted to the uppermost layers of the culture.

**Effects of EGF on HPV Expression.** Yasumoto et al. (27) recently reported that, in immortalized epidermal foreskin keratinocytes, EGF down-regulates HPV 16 E6/E7 transcription through an EGF-responsive silencer in the viral regulatory region. We asked whether addition of EGF after a period of EGF starvation affected the transcription of the resident HPV 6 or HPV 11 in the papilloma cells (Fig. 3). Relative amounts of HPV 6/11 mRNA present in the raft cultures varied with the EGF concentration. In this one experiment we had sufficient cells to include a steady-state culture without EGF starvation. There was a 14-fold increase in viral RNA when the EGF was removed for 5 days. Refeeding with 1 ng/ml EGF after 48 h reduced the viral RNA level by 40% relative to the 0 EGF culture, and 50 ng/ml reduced it by 81%. Five additional experiments evaluated the effects of withholding and then replacing EGF on steady-state levels of viral RNA. RNA levels in cultures with no EGF for 5 days were set at 1.0. Cultures fed with 1 ng/ml EGF after a 48-h period of deprivation showed a reduction in HPV RNA level to a mean value of 0.47, with a SD of ±0.17. This suggests that the starvation effect is partially reversible. The results

Fig. 2. Effects of EGF on the differentiation of papilloma cells. Papilloma cells were cultured on organotypic rafts and exposed to varying concentrations of EGF for the last 5 days of culture, and frozen sections were used for immunohistochemical detection of the differentiation-specific keratin 13 as described in “Materials and Methods.” A, 1 ng/ml EGF; B, 0 ng/ml EGF; C, 0 ng/ml EGF for 48 h, then 1 ng/ml for 72 h; D, 0 ng/ml EGF for 48 h, then 50 ng/ml for 72 h. Bar, 50 μm.
The cells were grown in serum-free medium in low calcium as a control for RNA loading. However, this is an abnormal differentiation. papilloma cells do undergo a differentiation process, as measured by involucrin synthesis (4). This reduction was specific and did not represent a general reduction in mRNA levels, since there was increased expression from the SV40 enhancer-minus construct under any of the conditions.

DISCUSSION

We have shown that cells derived from laryngeal papillomas showed increased staining of the EGFR, suggesting higher levels on the surface of the cells. The cells responded to EGF in the medium by a modest increase in proliferation, a striking reduction in K13 expression as a marker of normal differentiation, and a reduction in viral expression. However, only the differentiation response distinguished the papilloma cells from uninfected laryngeal cells.

Presence of high levels of EGFR on the papilloma cells did not result in increased proliferation. This is consistent with the low mitotic index of papillomas in vivo. Proliferative responses to EGF were less than anticipated in both types of cells, and basal proliferation was lower. There could be several reasons for this. The serum-free medium used for these studies contains small amounts of pituitary extract, which could provide growth factors. Alternatively, both normal keratinocytes and a number of tumor lines have been shown to synthesize and respond to transforming growth factor α, which binds to and activates the EGFR, resulting in autocrine growth stimulation (14, 36).

Induction of the normal differentiation pathway after removal of EGF was much more striking. We have previously shown that the papilloma cells do undergo a differentiation process, as measured by involucrin synthesis (4). However, this is an abnormal differentiation, with K13 expression delayed or missing. Now we have found that in the absence of added EGF, the papilloma cells showed levels of K13 staining comparable to that of uninfected cells cultured in the presence of EGF (5) or of normal tissue in vivo (4). The differentiation data, combined with the high EGFR levels, suggest that overresponsiveness to EGF might be a critical factor in the transformed phenotype.

In normal basal cells, EGFR levels and EGF response are regulated in part by internalization and degradation of receptor after binding EGF. Ligand binding eventually results in DNA synthesis and cell division. Movement of basal cells to a suprabasal position is accompanied by several events, including commitment to differentiation, loss of EGF, and synthesis of K13.

The papilloma cells maintained high levels of receptor on both basal and suprabasal cells. Binding of EGF had a minimal effect on the steady-state levels of receptor. Stimulation of these receptors did not result in DNA synthesis at levels greater than those seen in uninfected cells. However, it did block the differentiation of the suprabasal cells by the normal pathway. It does not block the abnormal pathway, since the cells do synthesize involucrin (4, 6).

EGF binding also had negative effects on viral expression. There was a consistent decrease in viral RNA when EGF was returned to the medium and decreased transcription from the HPV 11 E6 promoter in a transient expression assay. The regulatory mechanism(s) of these altered transcription levels is not known. There is a relationship between HPV expression and differentiation in vivo (37). Viral mRNA is expressed in larger amounts in suprabasal cells, and viral proliferation is limited to the upper layers of the epithelium. Therefore, in raft cultures, changes in viral expression could reflect alterations in cellular factors associated with normal differentiation. However, the transient assays were carried out with monolayer cultures of uninfected, undifferentiated cells in low-calcium medium. Nevertheless, there was a consistent reduction in expression from the HPV 11 enhancer/promoter when EGF was present.

The role of HPV 11 in the increased levels of the EGFR and in the inhibition of K13 expression after binding of EGF to the receptor must still be determined. Investigators have shown that the E5 gene products of HPV 6 or 16 and the similar E5 gene from bovine papillomavirus are able to induce transformation (25, 38–41). These E5 proteins activate the endogenous platelet-derived growth factor receptor (39) and transfected EGFR (40) in murine fibroblasts. Martin et al. (40)
found that cooperation between BPV E5 and the EGF receptors resulted in the inhibition of receptor degradation and the persistence of activated receptors on the surface of the cells. However, Pim et al. (25) found enhanced transformation with the addition of EGF to the medium but no evidence for either increased numbers of EGFR or changes in turnover rate in cells expressing HPV 16 E5 and EGFR.

Perhaps conflicting results in previous studies reflect differences in host cells, levels of proteins expressed from transfected plasmids, the papillomavirus type, or culture conditions. It is possible that HPV can induce phenotypic transformation in several ways: activation of receptors, overexpression of receptors, decreased receptor degradation, or all of these. We are currently investigating these possibilities, to understand the early events involved in the formation of these tumors.

REFERENCES


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