Increased Levels and Constitutive Tyrosine Phosphorylation of the Epidermal Growth Factor Receptor Contribute to Autonomous Growth of Human Papillomavirus Type 16 Immortalized Human Keratinocytes

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Abstract
Transfection of individual normal human foreskin keratinocyte (HKc) strains with human papillomavirus type 16 (HPV16) DNA results in the establishment of immortalized cell lines (HKc/HPV16) which, like normal HKc, require epidermal growth factor (EGF) and bovine pituitary extract (BPE) for proliferation in serum-free media. However, sublines which proliferate in serum-free media in the absence of EGF and BPE can be reproducibly established from individual HKc/HPV16 lines, following selection in serum-free media lacking EGF and BPE. The growth factor-independent sublines (HKc/GFI) proliferate in the absence of EGF and BPE at the same rate and to the same extent as in medium supplemented with these growth factors, whereas the parental HKc/HPV16 lines proliferate poorly in the absence of EGF and BPE. As a first approach to understanding the molecular basis by which HKc/GFI have lost their requirement for EGF, we compared EGF uptake and EGF receptor (EGFR) numbers in normal HKc, HKc/HPV16, and HKc/GFI. HKc/GFI exhibit increased EGF uptake and increased EGFR numbers compared to HKc/HPV16. A neutralizing antibody against the extracellular domain of the EGFR dramatically inhibited clonal growth of HKc/GFI, indicating that signaling through the EGFR must be important for the ability of HKc/GFI to proliferate in the absence of EGF. In addition, while in the absence of EGF normal HKc and HKc/HPV16 exhibited no detectable EGFR tyrosine phosphorylation, the EGFRs in HKc/GFI were tyrosine phosphorylated in the absence of EGF and hyperphosphorylated in the presence of EGF. Although an anti-TGF-α antibody inhibited the growth of HKc/GFI, we unexpectedly found that HKc/GFI and HKc/HPV16 secreted comparable and extremely low amounts of TGF-α (4 to 9 pg/10⁶ cells per 24 h); about 100- to 250-fold less than normal HKc (1018 pg/10⁶ cells per 24 h). No other ligands for the EGFR were detected in media conditioned by normal HKc, HKc/HPV16, or HKc/GFI. Thus, while overexpression and constitutive activation of the EGFR appear to be important features of HKc/GFI, enhanced secretion of TGF-α or other ligands for the EGFR does not explain the proliferation of HKc/GFI in the absence of EGF and BPE.

Introduction
An etiological role for HPV³, especially HPV types 16 and 18, in the genesis of human anogenital cancers, including cervical cancer, is generally accepted (1). Consistent with this view is the finding that transfection of normal HKc with HPV16 DNA results in the establishment of immortalized HKc/HPV16 cell lines (2–6). We have established several independently derived cell lines, HKc/HPV16-1 to -9, each by transfection of a plasmid containing a head-to-tail dimer of HPV16 DNA into normal HKc obtained from a single individual (2, 3). HKc/HPV16, like normal HKc, require EGF and BPE for rapid proliferation in serum-free MCDB153-LB medium. We initially reported that selection of the HKc/HPV16d-1 line in medium lacking EGF and BPE resulted in the establishment of HKc/GFI sublines which were able to proliferate in the absence of EGF and BPE with the same doubling time as their parental line cultured in medium containing these growth supplements (3). To investigate whether the acquisition of the GFI phenotype was a reproducible event in the in vitro progression of HPV16-immortalized cell lines, we attempted selection of GFI lines from four additional HKc/HPV16 lines (HKc/HPV16d-2 to -5), each derived from a single individual, and successfully established GFI sublines from all of them.

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© The abbreviations used are: HPV, human papillomavirus; HKc, human foreskin keratinocytes; HKc/HPV16, human keratinocytes immortalized by human papillomavirus type 16 DNA; EGF, epidermal growth factor; BPE, bovine pituitary extract; HKc/GFI, human keratinocyte lines which proliferate in the absence of EGF and BPE; GFI, growth factor independent; TGF, transforming growth factor; PDGF, platelet-derived growth factor; EGFR, epidermal growth factor receptor; QTDM, growth factor-depleted medium (MCDB153-LB medium lacking EGF and BPE); CM, complete medium; BPV-1, bovine papillomavirus type 1; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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Whereas a common feature of transformed cells is a reduced requirement for growth factors (7–15), the biochemical mechanisms leading to reduced growth factor dependence are unclear. In some cases, transformed cells appear to be producing their own polypeptide growth factors which allow the cells to proliferate through an autocrine mechanism (16). In fact, a number of polypeptide growth factors, including TGF-α (7, 9–11, 17), TGF-β (10), PDGF (10), and insulin-like growth factors I (9, 18) and II (19), have been identified in conditioned media collected from a variety of neoplastic cells. In some cases, it has been demonstrated that the autocrine growth factors are essential for the rapid proliferation of the transformed cells.

In addition to the production of autocrine growth factors, a growth advantage for transformed cells appears to have been obtained in some cases by the overexpression of growth factor receptors. For example, overexpression of the EGFR or EGF mRNAs has been frequently observed in a variety of human tumors and tumor cell lines (20–22). In general, elevated expression of the EGFR is linked to amplification and/or rearrangement of the EGFR gene (23–27). However, it is possible that mechanisms other than gene amplification may also lead to enhanced EGFR expression.

The EGFR, a member of the receptor tyrosine kinase family, is a M, 170,000 transmembrane glycoprotein containing an extracellular ligand recognition domain, a single hydrophobic membrane spanning domain, and a cytoplasmic protein tyrosine kinase domain (for reviews on the EGFR and receptor tyrosine kinases, see Refs. 28–31). The binding of EGF to the extracellular domain of the EGFR activates the tyrosine kinase activity of the cytoplasmic domain, leading to phosphorylation of both exogenous substrates and the receptor itself (autophosphorylation). The realization that several oncogenes represent altered cellular receptor tyrosine kinases or ligands for growth factor receptors underscores the importance of these tyrosine kinases in regulating normal cellular proliferation (29). It would appear that any alteration which leads to a loss of the normal regulatory constraints on the kinase domain of growth factor receptors would lead to constitutive activation of the receptor and could result in cellular transformation. In fact, some receptor tyrosine kinases were first identified as retroviral oncogenes (29). For example, the receptor-like protein encoded by the rat neu oncogene, which is highly homologous to the EGFR, contains a single point mutation in the transmembrane domain that results in a receptor whose intrinsic tyrosine kinase is constitutively activated (32, 33). However, amino acid substitutions in the transmembrane domain of the EGFR analogous to those present in the neu oncogene do not result in constitutive activation of the EGFR (34, 35), and no evidence for this mutation has been found in the human counterpart of the rat neu gene, erbB-2 (HER-2), in human breast and ovarian cancers (36). However, erbB-2 is often amplified and overexpressed in human tumors (36).

HPV16 is often found associated with squamous cell carcinomas of the human cervix. In addition, cervical cancers often express high levels of the EGFR (37, 38), and a recent report demonstrated increased EGFR levels in HPV16-immortalized human ectocervical cells (39). The levels of expression of the EGFR in clones of the vulvar carcinoma cell line A431 correlate with the proliferation rate of these cells (40). We therefore reasoned that enhanced EGFR expression and/or the secretion of TGF-α, an autocrine ligand for the EGFR, may play an important role in the ability of HKc/GFI to proliferate in the absence of EGF. In addition, enhanced tyrosine phosphorylation and constitutive activation of the EGFR in HKc/GFI could also contribute to autonomous growth. We therefore determined EGFR uptake, EGFR number, and the EGFR tyrosine phosphorylation state, before and after exposure to EGF, in HKc/GFI and in the parental HKc/HPV16 lines from which they were derived. We also analyzed media conditioned by normal HKc, HKc/HPV16, and HKc/GFI for the presence of TGF-α, EGF, and other ligands for the EGFR.

In this report, we demonstrate that HKc/GFI display enhanced EGF uptake and increased numbers of EGFRs. In addition, neutralizing antibodies against the extracellular domain of the EGFR inhibit the growth of HKc/GFI in the absence of exogenous EGF, suggesting that the EGFR is important to sustain the autonomous proliferation of HKc/GFI. Furthermore, the EGFRs of HKc/GFI exhibit detectable levels of tyrosine phosphorylation in the absence of EGF and are hyperphosphorylated in the presence of EGF. However, HKc/GFI secrete much lower levels of TGF-α than normal HKc, indicating that the ability of HKc/GFI to grow autonomously is not through a classical autocrine loop involving a secreted ligand for the EGFR.

**Results**

**Establishment of HKc/GFI Lines.** We previously established several independent HPV16 immortalized human keratinocyte lines, HKc/HPV16d-1 to -9, by transfecting normal foreskin HKc strains, each derived from a single individual, with a plasmid containing a head-to-tail dimer of the full length HPV16 genome (2, 3). Normal HKc, as well as the HKc/HPV16 lines at low population doublings, require EGF and BPE for proliferation and passage in serum-free MCD8153-LB medium (3). However, following prolonged culture, the growth factor requirements of HKc/ HPV16 became less stringent. We have reported the establishment of a subline of HKc/HPV16d-1, HKc/GFl-1, that was able to proliferate in MCD8153-LB medium lacking EGF and BPE (GDFM) with the same doubling time (about 36 h) as the parental line in medium containing EGF and BPE (CM; Ref. 3). HKc/GFl-1 originated as a few colonies of slowly growing cells in dishes maintained in GDFM for 2 months. The colonies were subcultured in GDFM, and their growth rate progressively improved to reach that of the parental HKc/HPV16d-1 line cultured in CM. We have now used the same selection protocol, maintenance in GDFM, to establish HKc/GFI lines from HKc/ HPV16d-2 to -5. All attempts to grow normal HKc in GDFM have failed, indicating that immortalization of normal HKc with HPV16 DNA is required for the HKc/GFI phenotype. On the other hand, all attempts to select HKc/GFI lines from HKc/HPV16 have proven successful, even when the parental cell line was monoclonal in origin (HKc/HPV16d-4).

**Comparison of the Growth Properties of Normal HKc, HKc/HPV16, and HKc/GFI in Complete Medium and Growth Factor-depleted Medium.** We compared the growth of HKc/HPV16 and HKc/GFI in CM and GDFM when cultured at either clonal density or in mass culture. As shown in Fig. 1, HKc/HPV16 form colonies at clonal density when maintained in CM but fail to grow significantly at clonal density in GDFM. However, the clonal growth of HKc/GFl-4 and HKc/GFl-5 in GDFM is comparable to that observed in CM (Fig. 1). HKc/GFl-2 and HKc/GFl-3 did not proliferate well when plated at low density, such as
in clonal growth assays, in either CM or GFDM (data not shown). The clonal growth assays were quantified using an image analysis system which determines the area of the culture dishes occupied by colonies. This analysis confirmed that HKc/GFI formed colonies that occupied approximately equivalent areas of the dish in both CM and GFDM (Fig. 2). HKc/GFI still require insulin for clonal growth in GFDM (Fig. 3).

Mass culture growth experiments were conducted to compare the growth kinetics of normal HKc, HKc/HPV16, and HKc/GFI cultured at high density in either CM or GFDM. Normal HKc fail to proliferate in GFDM (Fig. 4). In addition, HKc/HPV16 proliferate poorly in GFDM (Fig. 5, A and C). However, HKc/GFI exhibit similar mass culture growth kinetics in GFDM and CM (Fig. 5, B and D). Mass culture growth experiments with the other HKc/HPV16 and HKc/GFI lines have yielded similar results (data not shown).

**EGF Uptake by Normal HKc, HKc/HPV16, and HKc/GFI Cultured in CM and GFDM.** To examine if an increased ability to bind and internalize ligands through the EGFR contributed to the autonomous growth of HKc/GFI, we compared the uptake of $^{125}$I-EGF by normal HKc, HKc/HPV16, and HKc/GFI, all maintained in GFDM for at least 48 h prior to the assay. In general, HKc/HPV16 and HKc/GFI lines exhibited enhanced $^{125}$I-EGF uptake compared to normal HKc (Fig. 6A). In addition, the HKc/GFI lines took up substantially more $^{125}$I-EGF than the parental HKc/HPV16 lines from which they were selected (from 2- to 12-fold). We next measured $^{125}$I-EGF uptake in normal HKc, and two of the HKc/HPV16 and HKc/GFI lines cultured in CM (which contains 5 ng/ml EGF) to determine if HKc/GFI also exhibited increased EGF uptake under conditions in which the EGFR is down-regulated. As shown in Fig. 6B, the HKc/GFI lines still maintained enhanced $^{125}$I-EGF uptake (5- to 7-fold) over their parental HKc/HPV16 lines even when cultured in the presence of EGF. In addition, HKc/HPV16 and HKc/GFI exhibited substantially higher $^{125}$I-EGF uptake than normal HKc when cultured in CM (Fig. 6B).
EGFR Number and Affinity in HKc/HPV16 and HKc/GFI. The studies described above showed that HKc/GFI exhibit enhanced $^{125}$I-EGF uptake over the HKc/HPV16 lines from which they were derived. To determine if HKc/GFI had increased numbers of EGFRs compared to their parental HKc/HPV16 lines, we calculated the number of EGFRs per cell and receptor affinity by Scatchard analysis (41) of equilibrium EGF binding at 4°C to cells maintained in GFDM. HKc/GFI exhibited an increased number of EGFRs compared to their parental HKc/HPV16 lines (Table 1). HKc/GFId-4, which displayed the highest rate of EGF uptake also exhibited the highest number of EGFRs. There was no significant difference in the affinity of the EGFR for EGF (average $K_d$ of 0.5 nM) between HKc/HPV16 and HKc/GFI (Table 1).

In addition, we performed Western blot analysis with polyclonal anti-EGFR antibodies of cell extracts prepared from normal HKc, HKc/HPV16d-4, and HKc/GFId-4 incubated in the absence of EGF or exposed to EGF for 30 min. The Western blot showed that the HKc/HPV16 exhibited increased EGFRs compared to normal HKc and that the EGFR levels were further increased in HKc/GFI (Fig. 7). Overall, the Western data confirmed that EGFR levels are increased in HKc/GFI compared to their parental HKc/HPV16.

A Monoclonal Anti-EGFR Antibody Inhibits Clonal Growth of HKc/GFI in Growth Factor-depleted Medium. The increased EGFR levels in HKc/GFI suggested that the EGFR may be important for the ability of these cells to proliferate in the absence of EGF and BPE. To explore this possibility, we determined the ability of HKc/GFId-4 and HKc/GFId-5 to proliferate at clonal density in GFDM containing increasing amounts of a neutralizing anti-human EGFR monoclonal antibody directed against the extracellular domain of the human EGFR. The anti-EGFR antibody
inhibited, in a dose-dependent manner, the proliferation of HKc/GFId-4 (Figs. 8 and 9) and HKc/GFId-5 (Fig. 9). As a negative control, we used polyclonal sheep anti-human EGFR antibodies directed against the cytoplasmic domain of the receptor. As expected, these antibodies failed to inhibit growth of HKc/GFId-4 at clonal density (Fig. 9). The blocking anti-EGFR antibody against the extracellular domain of the EGFR also inhibited the growth of HKc/GFId-2 and HKc/GFId-3 when plated at mass culture density (10,000 cells/well) in 24-well plates (data not shown). Thus, despite the fact that HKc/GFI are cultured in the absence of EGF, EGFRs appear to be necessary to support cell proliferation in GFDM.

**EGFRs of HKc/GFI Are Constitutively Tyrosine Phosphorylated.** The phosphorylation of tyrosine residues within the cytoplasmic domain of the EGFR plays a critical role in modulating EGFR tyrosine kinase activity and signal transduction. Therefore, we determined by Western blot analysis, using a monoclonal anti-phosphotyrosine antibody, the phosphotyrosine content of the EGFR in cell lysates prepared from normal HKc, HKc/HPV16d-4, and HKc/GFId-4 incubated in the absence of EGF or after exposure to EGF for 30 min. Preliminary Western blot analysis of the same samples with anti-EGFR antibodies had shown that HKc/HPV16 and HKc/GFI had increased EGFR levels compared to normal HKc (Fig. 7). We therefore used this blot to estimate the relative EGFR concentrations in the samples. We then performed an additional Western blot, loaded with approximately equal EGFRs, using the anti-EGFR antibodies to confirm equal EGFR loading and to localize the EGFR band (not shown). A parallel blot was then probed with a monoclonal anti-phosphotyrosine antibody. Only EGFRs from HKc/GFId-4 exhibited tyrosine phosphorylation in the absence of EGFR treatment (Fig. 10). In fact, the constitutive phosphotyrosine level of EGFRs in HKc/GFId-4 was comparable to that observed in normal HKc in the presence of EGF. In addition, tyrosine phosphorylation levels of the EGFR in response to EGF were greater in HKc/HPV16d-4 than in normal HKc and much higher in HKc/GFId-4 than in HKc/HPV16d-4 (Fig. 10).

**HKc/GFI Do Not Secrete Increased Levels of TGF-α.** The findings that EGFR levels were increased in HKc/GFI, that a blocking anti-EGFR antibody inhibited the growth of HKc/GFI, and that the EGFRs in HKc/GFI were tyrosine phosphorylated in the absence of EGF raised the possibility that HKc/GFI secrete TGF-α, a growth factor which binds to the EGFR. Therefore, we performed specific radioimmunoassays for TGF-α and EGF and a radioreceptor competition assay to detect and quantify ligands for the EGFR in conditioned media collected from normal HKc, HKc/HPV16d-4, and HKc/GFId-4. The levels of TGF-α secreted in the conditioned medium by HKc/HPV16d-4 and HKc/GFId-4 were low (4 to 9 pg/106 cells per 24 h), and no significant differences were observed between the two lines (Table 2). Surprisingly, the levels of TGF-α secreted by the immortal-
ized HKc lines were 100- to 250-fold less than that secreted by normal HKc (Table 2). No EGF was detected in conditioned media from HKc/GFI-d-4, HKc/HPV16-d-4, or normal HKc (data not shown), and the radioreceptor competition assays provided no evidence that other ligands for the EGFR were secreted (Table 2).

The low level of TGF-α protein secreted by HKc/HPV16 and HKc/GFI in comparison to normal HKc raises the possibility that TGF-α may be retained at the cell surface in the HPV16-immortalized lines as a membrane bound form that is biologically active. We therefore determined the ability of HKc/GFI-d-4 to proliferate at clonal density in GFDM containing increasing amounts of a monoclonal anti-TGF-α antibody. The antibody inhibited the clonal growth of HKc/GFI-d-4 by about 64% at 500 ng/ml (Fig. 11).

**Discussion**

The HKc/GFI cells used in these studies are human epithelial cell lines which can be reproducibly established by selection of HPV16-immortalized HKc in EGFr- and BPE-free media. We have previously shown that from the HKc/GFI lines, it is possible to select cells resistant to serum and calcium-induced differentiation (3), and DiPaolo et al. (42, 43) have shown that these differentiation-resistant cells become tumorigenic following transfection with either a viral ras oncogene or herpes virus type 2 DNA. It should be noted that HKc/HPV16 lines produced by cotransfection of normal HKc with HPV16 and v-ras sequences or transfected with v-ras at early passages after immortalization with HPV16 were nontumorigenic, indicating that the cells became susceptible to malignant conversion only after becoming GFI and serum/calcium resistant. Whereas serum/calcium-resistant cells can also be selected directly from HKc/HPV16, this selection is much more difficult and much slower than from HKc/GFI (3). Therefore, the GFI phenotype constitutes a defined and reproducible intermediate step in the multistep process of neoplastic transformation initiated by HPV16 in human genital epithelial cells in vitro. Whereas many cell lines with reduced growth factor or serum requirements have been described, these cell lines are usually derived from human cancers or from the transformation of rodent cells in culture (7-15). It has been extremely difficult to reproducibly transform human cells and to develop an in vitro system using human cells that will mimic the multistep process of carcinogenesis that occurs in vivo (44). However, using HKc, we have been able to identify distinct stages in the process of transformation initiated by HPV16. In the first step, normal HKc are immortalized by transfection with HPV16 DNA and these cells, HKc/HPV16, maintain growth factor requirements similar to those of normal HKc. In a second stage, HKc/GFI lines which exhibit a total loss of their requirement for EGF and BPE, are selected as a small fraction of the total HKc/HPV16 cell population. In subsequent steps, HKc/GFI give rise to serum/calcium-resistant cells, which can then become tumorigenic. The reproducibility of the HKc/HPV16 and HKc/GFI system and the availability of several independently derived HKc/GFI lines with similar characteristics allowed us to begin to investigate the molecular basis for the acquisition of autonomous growth, which is a frequent characteristic of human cancer cells (16-20) and has also been observed in HPV-immortalized human mammary epithelial cells (45).

We have demonstrated in this study that all HKc/GFI lines, although each to a different extent, exhibit increased EGF uptake and EGF number compared to their parental HKc/HPV16 lines. In addition, most of the HKc/HPV16 lines exhibit increased EGF uptake when compared to normal HKc. Increased levels of EGFRs are a common characteristic of human cancer cells and likely play an important role in the maintenance of autonomous growth by cancer cells in vitro and in vivo (21-27, 37, 38). We therefore explored whether the EGFR on HKc/GFI was important for the growth of these cells in EGFr-free medium. The inhibition of growth of HKc/GFI by the blocking anti-EGFR antibody indicates that the EGFR is important to support cell proliferation. This result raised the possibility that HKc/GFI produce their own growth factor(s) which stimulate cell growth through the EGFR. Previous studies have shown that the expression of TGF-α, a ligand for the EGFR, is often increased in human cancer cells (9-11, 17, 20, 21, 27). However, we found that media conditioned by HKc/GFI and HKc/HPV16 contained about the same levels of TGF-α. Surprisingly, the levels of TGF-α secreted by both HKc/HPV16 and HKc/GFI were substantially lower than those secreted by normal HKc. This raises the interesting possibility that increased levels of TGF-α are present at the cell surface of HKc/GFI and that stimulation of proliferation may not require secretion of TGF-α into the medium. In this regard, the TGF-α precursor expressed on the cell surface has been shown to bind to the EGFR on adjacent cells and lead to signal transduction (46). Interestingly, we found that an anti-TGF-α antibody inhibited the proliferation of HKc/GFI in GFDM.

The presence of cell surface EGFRs whose tyrosine kinase domain is constitutively activated would represent yet an-
Fig. 10. Phosphotyrosine content of EGFRs from normal HKc, HKc/HPV16d-4, and HKc/GFd-4. Normal HKc (NHKc), HKc/HPV16d-4, and HKc/GFd-4 were cultured to approximately three-fourths confluence in 100-mm dishes. The cells were then washed with PBS, refed with GFDM, and 24 h later refed with GFDM or GFDM containing EGF (10 ng/ml) for 30 min. The cells were then lysed in 1 ml of lysis buffer, and cell lysates were processed as described in "Materials and Methods." Aliquots of cell lysates containing approximately equal EGFR amounts, as determined by Western blot analysis for the EGFR, were fractionated on a 7.5% SDS-PAGE gel. The proteins were transferred by electroblotting onto an Immobilon membrane and probed with a monoclonal anti-phosphotyrosine antibody. Left, position of protein molecular size markers on the gel; right, position of the EGFR (170 kDa).

Table 2. TGF-α levels in conditioned media from normal HKc, HKc/HPV16, and HKc/GFI measured by radioimmunoassay (RIA) and by an EGFR competition assay (EGFRA).

<table>
<thead>
<tr>
<th>Cells</th>
<th>TGF-α (pg/10⁶ cells/24 h)</th>
<th>TGF-α and other EGFR ligands (EGFRA) (pg/10⁶ cells/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal HKc</td>
<td>1018</td>
<td>973</td>
</tr>
<tr>
<td>HKc/HPV16d-4</td>
<td>9</td>
<td>ND</td>
</tr>
<tr>
<td>HKc/GFd-4</td>
<td>4</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Based on TGF-α standards.
ND, levels were below the detection limits of the assay (50 pg/10⁶ cells/24 h).

other possible mechanism for the maintenance of autonomous growth by HKc/GFI. To assess the tyrosine phosphorylation state of the EGFR, we performed Western blot analysis with an anti-phosphotyrosine antibody, using cell extracts prepared from normal HKc, HKc/HPV16, and HKc/GFI incubated in the absence or in the presence of EGF. Only EGFRs from HKc/GFI exhibited tyrosine phosphorylation in the absence of EGF, and these receptors became hyperphosphorylated in response to EGF treatment. It is worth noting that the EGFR of HKc/GFI is by far the predominant tyrosine phosphorylated protein in the cell extract. The mechanism that leads to tyrosine phosphorylation of the EGFR in HKc/GFI in the absence of EGF remains to be determined. Possible mechanisms include, as discussed above, EGFR activation via cell-surface TGF-α on adjacent cells. In addition, the elevated numbers of EGFR on the cell surface of HKc/GFI may lead to receptor dimerization in the absence of ligand. In this regard, the neu oncoprotein, p185<sub>neu</sub>, but not its cellular counterpart p185<sub>C-neu</sub>, is organized at the plasma membrane in an aggregated state, similar to that found following EGF-induced aggregation of the EGFR (47). Constitutive aggregation of p185<sub>neu</sub> may mimic ligand binding and therefore lead to activation of the tyrosine kinase of p185<sub>neu</sub> in the absence of ligand (47).
An important question to be resolved concerns the mechanism by which EGFR steady state protein levels are elevated in HKc/GFI. Although EGFR gene amplification and rearrangement is a common feature of human tumors expressing increased levels of EGFRs (23, 24, 26, 27), the EGFR gene is not amplified in either HKc/HPV16 or HKc/GFI. In addition, mRNA levels for the EGFR in HKc/GFI are not substantially different from those in their HKc/HPV16 parental lines. These results suggest that the increase in EGFRs observed in HKc/GFI may result from changes at the translational or posttranslational level. Further studies are necessary to resolve this important issue.

Another point of interest is the possible role of HPV16 gene products themselves in contributing to the GFI phenotype. In particular, the product of the E5 open reading frame of BPV-1 has been shown to cooperate with human EGF Rs to induce cellular transformation in mouse NIH 3T3 cells as monitored by focal transformation of cells in monolayer culture and anchorage-independent growth in soft agar (48). The cooperation between BPV E5 and the EGFR resulted in an inhibition of EGFR degradation and an inhibition of receptor down regulation, allowing activated receptors to remain at the cell surface (48). Waters et al. (49) concluded that the overall effect of BPV E5 is to stabilize intact EGFR complexes which may alter mitogenic signaling of the receptor. More recently, it has been established that BPV E5 interacts with the cytoplasmic domain of the EGFR (50). In addition, the COOH-terminal domain of E5 interacts with a member of the α-adaptin family, possibly inhibiting its activity, and thus inhibiting endocytosis and down-regulation of the EGFR (51). BPV E5 also causes constitutive activation of the β-receptor for PDGF in E5-transformed fibroblasts, and the E5 protein and the PDGF receptor exist in a stable complex in these cells (52). More recent work using a gene transfer approach has provided further evidence that the PDGF receptor can mediate transformation by the BPV-1 E5 protein (53). BPV E5 specifically interacts with the transmembrane domain of the PDGF receptor (50). In addition, BPV-1 E5 binds to the M, 16,000 component of vacular H+-ATPases, the enzymes responsible for generating the acidic internal pH of endosomes (54, 55). Since the acidic environment of endosomes is required for the dissociation of ligand-receptor complexes, including EGF and PDGF from their respective receptors, an alteration of the activity of the vacular H+-ATPases by E5 could lead to a prolonged interaction between growth factors and their receptors and could also influence receptor recycling and/or turnover (55). Although BPV-1 E5 and HPV16 E5 proteins share little sequence homology, studies have indicated that both BPV-1 and HPV16 E5 can transform murine keratinocytes (56). The HPV16 E5 gene product also interacts with the EGFR and induces transformation of mouse cells, at least in part by enhancing signal transduction from the EGFR to the nucleus (57, 58). In addition, the HPV16 E5-expressing rodent cells displayed reduced serum requirements (58). Recent studies by Straight et al. (59) have shown that HPV16 E5 increases EGFR numbers in HKc and affects down-regulation of the EGFR, primarily by increasing the number of receptors which recycle back to the cell surface after ligand binding. Furthermore, like BPV-1 E5, HPV16 E5 associates with the M, 16,000 component of the vacular ATPases (60). We have recently found that HPV16 mRNA expression from the early open reading frames, including E5, is elevated in the HKc/GFI, compared to their parental HKc/HPV16 lines. Therefore, overexpression of E5 protein in HKc/GFI and cooperation between E5 and the EGFR may lead to constitutively active receptors that do not require ligand and thus result in cellular proliferation in the absence of growth factors. We are actively pursuing this possibility by testing cells immortalized by E6/E7 or by E7 alone for the GFI phenotype, both before and after transfection with E5-expressing plasmids.

The results presented in this paper give some insights into the possible mechanisms by which HKc/GFI proliferate in the absence of EGF. An explanation for the loss of requirement for BPE, which is a nondefined component of the medium, must await the identification of the growth factor(s) contained in this extract that are important for HKc proliferation.

Materials and Methods

Materials. 125I-EGF (105 μCi/μg) was from ICN Radiochemicals. A neutralizing monoclonal anti-human EGFR antibody directed against the extracellular domain of the EGFR (clone LA1), a polyclonal sheep anti-human EGFR antibody against the cytoplasmic domain of the receptor, and a monoclonal anti-phosphotyrosine antibody (clone 4G10) were purchased from Upstate Biotechnology, Inc. A monoclonal anti-TGF-α antibody (clone 213–4.4) that recognizes residues 34–50 of TGF-α was purchased from Oncogene Science. A rabbit anti-sheep IgG alkaline phosphatase-conjugated antibody, a goat anti-mouse IgG alkaline phosphatase-conjugated antibody, and SDS-PAGE reagents were purchased from Bio-Rad. Immobilization hybridization membrane was from Millipore. Aprotinin was from Miles, Inc. or Sigma Chemical Co., and soybean trypsin inhibitor was from Sigma.

Cells and Cell Culture Conditions. Normal HKc were isolated from newborn foreskins as described previously (2, 3) except the epidermis was separated from the dermis by incubation overnight at 4°C in 0.25% trypsin (Gibco Laboratories Life Technologies) instead of collagenase. Isolation and characterization of the immortalized HKc/HPV16 lines have been described in detail in previous publications (2, 3). These cell lines were obtained by transfecting normal HKc strains, each derived from a different individual, with a plasmid containing a head-to-tail dimer of the full length HPV16 DNA cloned into the BamHI site of the vector pDMMTneo (2, 3). The different immortalized lines used in the present study were selected with G418 and were designated HKc/HPV16d-1 to -5. Both normal HKc and HKc/HPV16 were cultured in serum-free MCDB153-LB medium, supplemented with hydrocortisone (0.2 μM), insulin (5 μg/ml), transferrin (10 μg/ml), triiodothyronine (10 nM), CaCl₂ (0.1 mEq/l), EGF (5 ng/ml), and BPE (35–50 μg protein/ml) with medium changes every 48 h (1, 2). This medium is referred to as CM.

HKc/GFI were established by selection of the various HKc/HPV16 lines in CM lacking EGF and BPE (GFDM). The establishment and some of the properties of the first GFI line, HKc/GF1d-1, have been described previously (3). All HKc/GFI lines were routinely maintained in GFDM.

Clonal Growth Assays. HKc/HPV16 and HKc/GFI were plated at low density (2,000–4,000 cells/dish) in 60-mm dishes in either CM or GFDM. Cells were fed 1 and 6 days.

5 L. L. Zyazk, L. Pirisi, and K. E. Creek, unpublished results.
after plating with 8 ml/dish of either CM or GFDM. Colonies were fixed in methanol and stained with Giemsa 10–12 days after plating. The total area of the colonies, relative to the area of the dish, was measured by computerized image analysis (Image I).

**Mass Culture Growth Assays.** Normal HKc, HKc/HPV16, and HKc/GFI (20,000 dish) were plated in 35-mm dishes in either CM or GFDM and refed 24 h after plating and every 48 h thereafter. Proliferation in CM and in GFDM was monitored by trypsinizing and counting cells from 3 dishes/time point per condition in a hemocytometer at various times after plating.

**EGF Uptake Assays.** Cells were plated at 40,000 cells/35-mm dish and refed as indicated 24 h later with subsequent media changes every 24 to 48 h. Every day until the cells reached confluence, three dishes/time point were used for EGF uptake assays. Cells were washed two times at 4°C with ice-cold GFDM containing 0.1% BSA. 125I-EGF (1.0 nM) was then added to each dish in 1 ml of the same medium. The cells were then incubated at 37°C for 1 h. After aspirating the medium, cells were washed four times with PBS containing 0.1% BSA (1 ml per wash) and then scraped into 1 ml of 0.1 M NaOH containing 0.1% SDS. Radioactivity in samples (0.75–0.85 ml) of the solubilized cells was determined in a Beckman 4000 gamma counter. Nonspecific binding was determined in parallel cultures incubated with a 75-fold molar excess of unlabeled EGF. Nonspecific binding was less than 10% of total binding. Cell counts were performed in three parallel dishes harvested at the same time.

**EGF Binding Assays.** Cells were plated at approximately 40,000 cells/35-mm dish in CM and then refed with GFDM 24 h after plating. At a density of approximately 200,000 cells/dish, cells were assayed for binding of 125I-EGF at 4°C. All cells were washed twice with GFDM containing 0.1% BSA. 125I-EGF (0.15 μCi) was added to each dish in GFDM (1.0 ml) containing 0.1% BSA, along with varying amounts of unlabeled EGF (up to 10.0 nM). Cells were incubated at 4°C for 2 h, washed 4 times (1 ml per wash) in PBS containing 0.1% BSA, and scraped into 1 ml of 0.1 M NaOH containing 0.1% SDS. Samples were counted in a Beckman 4000 gamma counter. Nonspecific binding was determined in cultures containing a 75-fold molar excess of unlabeled EGF. Nonspecific binding was less than 10% of total binding. The data were analyzed by the method of Scatchard (41). All binding assays were performed in triplicate.

**Western Blot Analysis for the EGF and Phosphotyrosine.** Normal HKc, HKc/HPV16d-4, and HKc/GFI4d-4 were cultured in 100-mm tissue culture dishes until approximately three-fourths confluence. Cells were then washed with PBS and fed with GFDM for 24 h. The cells were then refed with fresh GFDM, with or without EGF (10 ng/ml), and incubated for 30 min. Cells were then washed twice with PBS and lysed in 1 ml/dish of lysis buffer (70 mM Tris-HCl (pH 6.8)-11% glycerol-2.2% SDS-2 mM Na2VO4-1 mM NaF). The cell lysates were transferred to Eppendorf tubes, boiled for 5 min, and clarified by centrifugation for 10 min at 12,000 × g at 4°C in an Eppendorf centrifuge. The cell lysates were stored at −80°C. The protein concentration of the cell lysates was determined using a detergent-compatible protein assay kit (Bio-Rad). Cell lysates were separated on 7.5% SDS-PAGE gels as described by Laemmli (61) and electrophoretically transferred to Immobilon membranes in 25 mM Tris-192 mM glycine-20% methanol using a Bio-Rad Trans-Blot apparatus at 30 V for 16 h, followed by 60 V for 2 h, or 100 V for 1 h (62). The blots were then incubated for 3 h at room temperature with either polyclonal sheep anti-human EGFR antibodies (1 μg/ml) or a monoclonal anti-phosphotyrosine antibody (1 μg/ml) and then washed following the manufacturers’ recommendations. The membranes were then incubated with alkaline phosphatase conjugates of the appropriate secondary antibodies for 3 h at room temperature and extensively washed. EGFR or phosphotyrosine-containing proteins were visualized using a Bio-Rad alkaline phosphatase substrate kit.

**Collection of Conditioned Media.** Normal HKc and HKc/HPV16 were cultured in CM until subconfluent. The medium was then switched to GFDM for 48 h; then the cells were refed with fresh GFDM, and conditioned media was collected 24 h later. HKc/GFI were maintained in GFDM until subconfluent with feedings every 48 h and then fed with fresh GFDM; conditioned media were collected 24 h later. Conditioned media were stored at −20°C. Cell numbers were determined by counting in a hemocytometer after trypsinization.

**TGF-α and EGF Radioimmunoassays and EGF Competition Assay.** Samples (30 ml) of conditioned media were thawed, dialyzed against deionized water at 4°C for 48 h in 3500-molecular weight cut off dialysis tubing (Spectrum Medical Industries), and lyophilized. Samples were then resuspended in 1.5 ml each of assay buffer (75 mM Tris-Cl (pH 8.0), 100 mM NaCl, 0.15% BSA, 1 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 0.5 mM EDTA, and 0.05% sodium azide). TGF-α and EGF were measured using specific radioimmunoassay kits from Biomedical Technologies, Inc. An EGF radioassay kit from Biomedical Technologies, Inc. was used to detect ligands present in conditioned media which compete with 125I-EGF for binding to stabilized EGFR. In this radioreceptor competition assay, EGF and 125I-EGF are mixed with unlabeled EGF standards (0.2–25 ng/ml) or conditioned media and incubated for 1 h at 37°C. The samples are then diluted with ice-cold buffer, and the EGFR are collected by centrifugation. The supernatant is discarded, and radioactivity associated with the EGFR is determined using a gamma counter.

**References**


