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Human Papillomavirus Type 16 E6 and E7 Cooperate to Increase Epidermal Growth Factor Receptor (EGFR) mRNA Levels, Overcoming Mechanisms by which Excessive EGFR Signaling Shortens the Life Span of Normal Human Keratinocytes[1, 2]

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ABSTRACT

Epidermal growth factor receptor (EGFR) levels are dramatically increased in human keratinocytes (HKc) immortalized with full-length human papillomavirus type 16 (HPV16) DNA (HKc/HPV16), but increases in EGFR levels actually precede immortalization. In some normal HKc strains, acute expression of HPV16 E6 (but not HPV16 E5, HPV16 E7, or HPV6 E6) from LXSN retroviral vectors produced an increase in EGFR mRNA levels detectable at 24 h and stable for up to 10 days after infection. However, about one-half of the individual normal HKc strains we analyzed proved unresponsive to E6 induction of EGFR mRNA despite the robust expression of E6 and degradation of p53. E6 responsiveness of normal HKc strains correlated inversely with initial EGFR levels: although HKc strains expressing relatively low basal EGFR levels grew poorly and tolerated the infection protocol with difficulty, they responded to E6 with an increase in EGFR mRNA and protein and with robust proliferation. However, those HKc strains expressing high basal EGFR levels grew well, but did not respond to E6 with increased EGFR levels or with proliferation. Immunostaining of paraffin-embedded foreskin tissue for the EGFR confirmed that there is an intrinsic interindividual variability of EGFR expression in HKc. These results prompted us to investigate the effects of overexpression of the EGFR in normal HKc. Infection of normal HKc with a LXSN retrovirus expressing the full-length human EGFR cDNA resulted in a dramatic reduction in growth rate and a shorter life span. Although acute expression (1–10 days after infection) of HPV16 E7 alone did not induce the EGFR, acute expression of E6 and E7 together increased EGFR levels in normal HKc unresponsive to E6 alone. Also, HKc infected with E7 alone expressed increased EGFR levels at early stages of extended life span (at passage 9 after infection), and HKc immortalized by HPV16 E7 alone expressed EGFR levels comparable with those of E6/E7-immortalized cells. These results support a key role of the EGFR in HPV16-mediated transformation of HKc. In addition, these data show that normal HKc do not tolerate excessive EGFR levels/signaling, and such intolerance must be overcome in order for HKc to become immortalized by HPV16. We conclude that both E6 and E7 contribute to increasing EGFR levels, but with different mechanisms: although E6 can increase EGFR levels, it cannot overcome the resistance of normal HKc to excessive EGFR signaling. On the other hand E7, which alone does not acutely increase EGFR mRNA or protein, allows for EGFR overexpression in normal HKc.

INTRODUCTION

Oncogenic HPV[4] is a major cause of cervical cancer and immortalizes HKc and cervical cells in culture (1–7). In our *in vitro* model system for HPV16-mediated carcinogenesis (3–5, 8), normal HKc transfected with HPV16 DNA undergo malignant transformation through a series of phenotypically well-defined "steps." Upon transfection with HPV16 DNA, normal HKc become immortalized, giving rise to stable lines (HKc/HPV16) that still retain growth requirements and differentiation properties similar to those of normal cells, and are not tumorigenic. Selection of HKc/HPV16 in serum-free MCDB153-LB medium devoid of EGF and BPE gives rise to EGF- and BPE-independent lines (HKc/GFI). HKc/GFI are still responsive to differentiation stimuli (serum, high calcium) and are not tumorigenic. HKc/GFI can be selected further in medium supplemented with serum and high calcium (>0.3 mM) to yield differentiation-resistant lines (HKc/DR). HKc/DR, but not HKc/HPV16, are susceptible to malignant conversion by either v-*ras* or HSV2 sequences (9, 10).

Previously we have shown that individual, independently derived HKc/HPV16 lines consistently exhibit higher EGF uptake and EGFR levels than normal HKc when cultured either in the presence or in the absence of exogenous EGF (8). The acquisition of the HKc/GFI phenotype is accompanied by an additional increase in EGFR protein levels. EGFR in HKc/GFI, but not HKc/HPV16 or normal HKc, are activated in the absence of EGF, and signaling through the EGFR is essential to maintain autonomous growth of HKc/GFI in the absence of EGF and BPE (8). The studies by Zyzak *et al.* (8) were conducted using well-established, immortal HKc/HPV16 lines in long-term culture and their respective HKc/GFI and HKc/DR derivatives. In the present study, we asked whether increases in EGFR can be detected early after the transfection of normal HKc with HPV16 DNA (which would exclude that long-term culturing is responsible for EGFR increases) and whether immortalization is necessary for this effect. In addition, we explore the possibility that acute expression of single HPV16 oncoproteins may produce an increase in EGFR in normal HKc. We show here that a robust increase in EGFR mRNA levels can be detected early after transfection of normal HKc with full-length HPV16 DNA, before the cells are immortalized. In addition, acute

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[4] The abbreviations used are: HPV, human papillomavirus; HKc, human keratinocytes; HKc/HPV16, human keratinocytes immortalized with HPV16; EGF, epidermal growth factor; BPE, bovine pituitary extract; HKc/GFI, growth-factor independent human keratinocytes; HKc/DR, differentiation-resistant human keratinocytes; EGFR, epidermal growth factor receptor; LB medium, Luria-Bertani medium; GFDM, growth factor-depleted medium, lacking EGF and BPE; RPA, ribonuclease protection assay; ORF, open reading frame; PD, population doublings; Rb, Retinoblastoma Gene Product.

[5] The unexpected bands of lower molecular weight produced by this EGFR probe with HKc/HPV16 and HKc/GFI mRNA (Fig. 1*A*) were mapped to two alternatively spliced EGFR mRNA forms. We have characterized these EGFR splice variants and found that their levels increase relative to the levels of wild-type EGFR mRNA in established HPV16-immortalized HKc but not in HKc acutely expressing HPV16 E6, E7, or E6 and E7 together (J. Liao, T. S. W. Engin, X. Xu, and L. Pirisi, manuscript in preparation).

expression of HPV16 E6 alone results in an increase in EGFR mRNA levels in normal HKc, whereas acute expression of HPV16 E7 alone or HPV16 E5 alone did not produce this effect. However, about one-half of the normal HKc strains we examined proved unresponsive to E6 induction of EGFR mRNA levels, despite the fact that E6 was expressed and functional in these cells. We determined that basal EGFR mRNA and protein levels vary greatly among individual HKc strains as well as among individual skin specimens. We observed that HKc strains that proved unresponsive to E6 exhibited high basal levels of EGFR, whereas the strains that did respond to E6 had relatively low basal EGFR expression. Therefore, we investigated the effects of overexpression of the EGFR in normal HKc and found that acute EGFR expression from an LXSN retrovirus leads to dramatically reduced growth rates and shortened life span in culture. Intriguingly, two HKc strains that did not respond to E6 alone exhibited higher EGFR mRNA levels when infected with E6 and E7 retroviruses together. In addition, despite the fact that acute expression of E7 alone does not induce the EGFR, cells infected with E7 alone exhibited increased EGFR mRNA levels at passage 9 after G418 selection, and HKc immortalized with E7 alone expressed levels of EGFR mRNA comparable with those of cells immortalized with E6 and E7 together. Our results suggest that increased EGFR expression plays an important role in the HPV16-mediated transformation of human epithelial cells. These observations also indicate that overexpression of the EGFR is not tolerated by normal HKc. Thus, to immortalize HKc, the HPV16 oncoproteins must not only increase EGFR levels, but also overcome the mechanisms that inhibit such increases in normal human epithelial cells. These two functions are carried out by E6 and E7, respectively.

MATERIALS AND METHODS

Cell Culture and Cell Lines. Normal HKc were isolated from neonatal foreskin and cultured in serum-free MCDB153-LB medium as described previously (3), except that trypsin (0.25% in Hanks' buffered saline, at 4°C, for 18–22 h) was used in place of collagenase to separate the epidermis from the dermis. The HKc/HPV16 and HKc/GFI lines used in this study have been described previously and characterized (3, 5, 8). To establish new HKc/HPV16 lines, the 7.9 kb HPV16 genome was purified after *Bam*HI digestion from the plasmid pMHPV16d (3) by gel electrophoresis and electroelution. The HPV16 DNA free of vector sequences (1 μg/dish, with 10 μg/dish of carrier calf thymus DNA) was then transfected into normal HKc at the first passage using lipofectin reagent (Life Technologies, Inc.), according to the protocol provided by the manufacturer. Transfected cells were passaged into 100-mm dishes and incubated until colonies of actively proliferating cells were detected. Colonies from each individual HKc strain were pooled, and plated again. RNA extraction was performed between 5 and 10 passages after transfection.

Northern Blot Analysis. Normal HKc and HKc/HPV16 were plated in complete MCDB153-LB medium, and HKc/GFI were plated in MCDB153-LB devoid of EGF and BPE (GFDM). Cells were allowed to grow to about 75% confluence, then washed with PBS and incubated in GFDM for at least 48 h. Total RNA was isolated by the guanidinium thiocyanate/cesium trifluoroacetate gradient centrifugation method (5) and subjected to Northern blot analysis with an EGFR cDNA probe (gift of Dr. Ira Pastan, National Cancer Institute, Bethesda, MD, USA). RNA loading was determined by hybridizing the blots with a β2-microglobulin cDNA probe (courtesy of Dr. Pedro Lazo, Hospital Universitario de la Princesa, Madrid, Spain). Probes were labeled by random priming, using a commercial kit (Promega) and [^{32}P]dCTP (Amersham Life Science Products). Northern blot analysis was conducted according to standard protocols, as described previously (5).

RNase Protection Analysis. Riboprobes synthesized using a commercial kit (Promega) from cloned templates corresponding to various regions (see below) of the EGFR cDNA were used to compare EGFR levels by RPA (Hybspeed RPA; Ambion). Ten μg of total RNA were hybridized with antisense probes (150,000 cpm) for EGFR and an internal standard (cyclophilin, β-actin or 28 s rRNA; Ambion) as a loading control. The RNase-digested products were resolved on a 5% polyacrylamide denaturing gel containing 8 M urea at 225 V for 3 h and exposed to X-ray film (Kodak) for 1–3 days. Two EGFR riboprobes were constructed on the template of the following regions of the human EGFR cDNA: probe V, nt 1321–1663, protected fragment size 343 bp; and probe IX, nt 2770–3205 protected fragment size 436 bp. All fragments were produced by PCR, cloned into the pGEM-T Easy vector (Promega), and sequenced.

Southern Blot Analysis. Genomic DNA (10 μg) from normal HKc, HKc/HPV16 and HKc/GFI was digested with *Hin*dIII (10 units/μg in a final volume of 200 μl) at 37°C for 12 h. The digests were separated on a 0.8% agarose gel and transferred to Gene Screen membrane (DuPont) according to the manufacturer's instructions. Southern blot analysis was conducted according to standard procedures (3, 5) with a human EGFR cDNA probe labeled with ^{32}P by random priming (Promega).

Assay for EGFR Tyrosine Kinase Activity. Normal HKc, HKc/HPV16d-4 and HKc/GFId-4 were cultured in 150-mm tissue culture dishes (20 each) in their respective media. At 75% confluence, cells were washed with PBS and media replaced with GFDM. Cells were refed with GFDM 24 h later for a total of 48 h in EGF-free medium. Cells were then washed in PBS containing 5 mM EDTA, scraped into the same buffer, and collected by centrifugation. Cell pellets were washed once in ice-cold PBS and lysed in 10 ml of ice-cold lysis buffer [50 mM HEPES (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF, 10% glycerol, 1% Triton X-100, 1% aprotinin, 2 mM sodium orthovanadate, and 1 mM sodium fluoride]. The cell lysates were incubated with rocking at 4°C for 20 min, their pH was adjusted to 8.5 with 1 N NaOH, and the lysates were then clarified by centrifugation at 3,000 $\times$ *g* for 10 min, and then at 10,000 $\times$ *g* for 1 h at 4°C. The clarified cell lysates were then applied to an affinity column (Amino-Link; Pierce) containing an immobilized anti-EGFR antibody directed against the extracellular domain of the EGFR (Upstate Biotechnologies, Inc.). After washing with lysis buffer, EGFR was eluted from the column with 0.1 M glycine pH 2.8. Fractions (8, of 0.9 ml each) were collected into Eppendorf tubes containing 100 μl of 0.5 M HEPES (pH 7.9), pooled, concentrated 16- to 20-fold using a Centricon 10 (Amicon), and EGFR content and purity were assessed by SDS-PAGE. The relative concentration of EGFR in the samples was estimated by Western blot analysis of various dilutions of each partially purified EGFR preparation with anti-EGFR antibodies. Tyrosine kinase activity in aliquots of partially purified EGFR containing approximately equal amounts of EGFR was measured by determining the transfer of ^{32}P from [γ-^{32}P]-ATP to angiotensin II, as described by Bertics and Gill (11).

Retroviral Vectors and Retroviral Infection Protocols. LXSN-based amphotropic retroviral vectors, kindly provided by Denise Galloway, were used to infect primary neonatal foreskin HKc as described previously (12). In addition, we subcloned into the pLXSN vector the entire coding sequence for the human EGFR from the plasmid pCO12 (kindly provided by Dr. John Schiller, National Cancer Institute, Bethesda, MD). The insert region in the resulting construct was sequenced, and the plasmid was transfected into PA317 cells by the calcium-phosphate precipitation method. After G418 selection and cloning, culture supernatants from individual PA317 clones were used to infect NIH 3T3 and CHO cells, which were then G418-selected and subjected to immunofluorescent staining for the human EGFR to ensure that the retrovirus elicited the expression of human EGFR. Virus stocks were then used to infect normal HKc.

Retroviral infection was performed according to published procedures (12), which were modified as follows. Primary HKc were infected with filtered (0.22-μm pore size) supernatant from an overnight culture of PA317 cells, producing the control LXSN virus or LSXN retroviruses encoding 6E6, 16E5, 16E6, 16E7, or 16E6/E7 ORFs of HPV or the LXSN-hEGFR virus. Virus stocks were diluted 1:4 in calcium-free complete MCDB153-LB medium before infection to reduce as much as possible their calcium and serum contents. Cells were passaged 24 h after infection and either selected in 50 μg/ml G418 for 4 days or harvested without G418 selection. In that case, parallel dishes were G418-selected to monitor infection efficiencies. RNA (Tri Reagent; Molecular Research Center) and protein extracts were collected at various times (1–10 days) after infection.

EGFR Immunostaining. Foreskin tissue was fixed in buffered formalin and paraffin-embedded according to standard procedures. Sections were deparaffinized in several changes of xylene, rehydrated in decreasing concentrations of ethanol in water, and washed in PBS containing blocking calf serum (1.5%).

Sections were then treated with 0.3% hydrogen peroxide to quench endogenous peroxidases and incubated with a 1:100 dilution of a monoclonal anti-EGFR antibody (GRO1; Oncogene Research Products) using the Vectastain Elite kit with the Vector VIP Substrate (Vector Laboratories) according to the manufacturers' instructions. Counterstaining of nuclei was performed with methyl green for 10 min at 60°C.

Western Blot Analysis. Total cell lysates (40 μg protein/sample) were resolved on a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose (Bio-Rad) membranes. The blots were incubated in blocking solution (2% nonfat dry milk) overnight at 4°C. Blots were then probed with an antihuman p53 monoclonal antibody (DO-1; Oncogene Research Products) at a concentration of 0.1 μg/ml. Blots were then probed with a 1:10,000 dilution of biotin-labeled goat antimouse secondary antibody (Sigma Chemical Co.). The membranes were then incubated in a 1:1,000 dilution of streptavidin-horseradish peroxidase conjugate (Amersham Life Science), and bands were visualized using the Supersignal chemiluminescence system (Pierce).

RESULTS

We first explored whether the increases in EGFR levels observed at the immortalization and HKc/GFI stages in our *in vitro* model system for HPV16-mediated multistep carcinogenesis (8) involved increases in mRNA levels for the EGFR. RPA of RNA extracted from normal HKc, HKc/HPV16, and HKc/GFI with a probe specific for the EGFR showed that although EGFR mRNA levels increase dramatically in HKc/HPV16 compared with normal HKc, there was no additional increase in HKc/GFI compared with their parental HKc/HPV16 line (Fig. 1*A*). This observation was repeated with four of four HKc/HPV16 lines and their respective HKc/GFI derivatives (data not shown). This result, together with our previous observation that protein levels for the EGFR increase up to 10-fold in HKc/GFI compared with their parental HKc/HPV16 lines (8) indicates that different mechanisms mediate the increase of EGFR levels at these two initial stages of HPV16-mediated transformation *in vitro*. Southern blot analysis of DNA extracted from normal HKc, HKc/HPV16, and HKc/GFI determined that EGFR increases were not attributable to gene amplification (Fig. 1*B*).

To further investigate EGFR function and responsiveness to EGF, we assayed tyrosine kinase activity of partially purified EGFRs from normal HKc, HKc/HPV16d-4, and HKc/GFId-4 after a 48-h incubation in the absence of EGF and BPE. EGFRs were partially purified by chromatography on anti-EGFR immuno-affinity columns and concentrated as described in "Materials and Methods." The relative concentration of EGFR in the concentrated fractions was estimated by Western blot analysis with anti-EGFR antibodies as described previously (8), and aliquots of the purified fractions containing approximately equal amounts of EGFR were assayed for tyrosine kinase activity, with or without a 15-min treatment with 25 nM EGF. Tyrosine kinase activity was assayed by measuring the transfer of radioactive phosphate from [γ-^{32}P]ATP to angiotensin II (11). As shown in Fig. 1*C*, there was no constitutive tyrosine kinase activity in EGFR isolated from either normal HKc or HKc/HPV16, and in both these cell types, EGF produced a response of approximately the same magnitude. HKc/GFI exhibited robust tyrosine kinase activity in the absence of EGF comparable with the levels observed in normal HKc and HKc/HPV16 stimulated with EGF. Tyrosine kinase activity in HKc/GFI was increased further by EGF treatment. We conclude from this experiment that the EGFRs in HKc/HPV16 at early stages of *in vitro* progression function very similarly to those in normal HKc with no major alterations of EGF response. Constitutive tyrosine kinase activity (in the absence of EGF) and a strong response to EGF ensue at later stages of *in vitro* progression in HKc/GFI.

To confirm that the increase in EGFR mRNA expression in HKc/HPV16 lines, compared with normal HKc, was directly associated

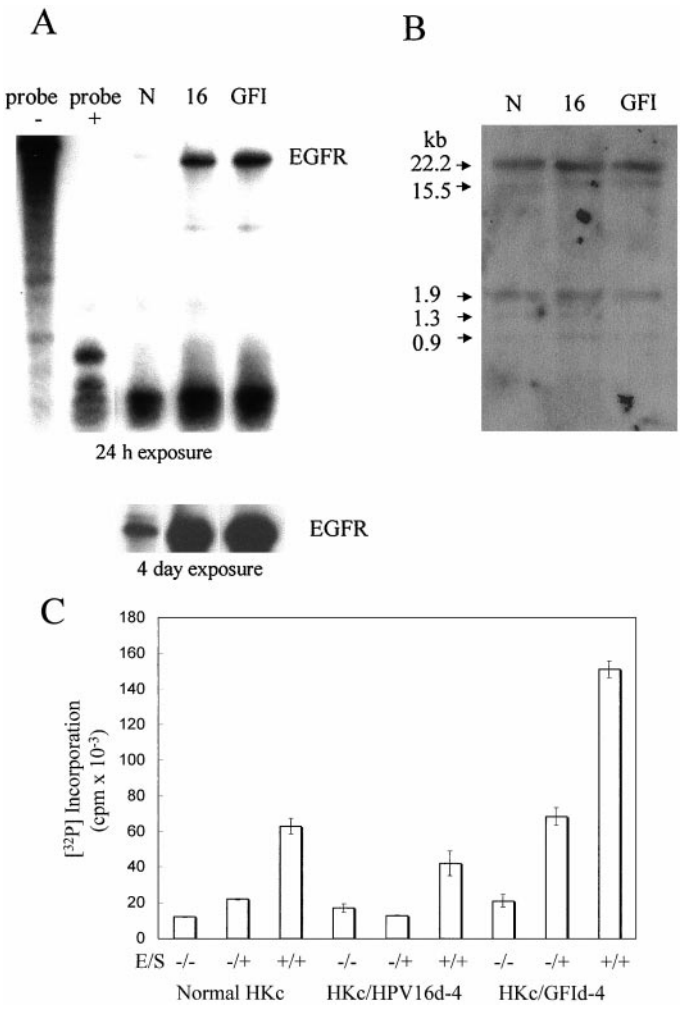

Fig. 1. *A*, RNase protection analysis of EGFR expression in normal and transformed human keratinocytes. Total RNA was isolated from normal HKc at passage 1 (*N*), HKc/HPV16d-4 (16), and HKc/GFId-4 (*GFI*). An antisense riboprobe specific for the EGFR (probe V; see "Materials and Methods") was used to compare total EGFR mRNA levels (5). A 24-h and a 4-day exposure are shown. *B*, Southern blot analysis of genomic DNA from normal (*N*), HKc/HPV16d-4 (16), and HKc/GFId-4 (*GFI*) digested with *Hind*III and probed for EGFR as described in "Materials and Methods." *C*, tyrosine kinase activity of partially purified EGFR from normal HKc, HKc/HPV16d-4, and HKc/GFId-4. *E/S*, EGF/substrate (angiotensin II).

with HPV16 gene expression and not just a consequence of long-term culturing, we established several new HKc/HPV16 lines from individual normal HKc strains. We transfected each normal HKc strain with *Bam*HI-linearized, gel-purified, full-length HPV16 DNA free of vector sequences. RNA was extracted from each newly derived HKc/HPV16 line as soon as the line was established, but before any appearance of crisis, between 5 and 10 passages after transfection. RNA was also extracted from each individual normal HKc strain of origin at the second passage, when cells were still actively proliferating at a growth rate comparable with that of the established HKc/HPV16 lines. Each HKc/HPV16 line was then compared with its normal HKc strain of origin for EGFR expression by Northern blot analysis, because we intended to determine the size and distribution of the EGFR messages produced as well as their levels. Specific EGFR messages of 10.2 and 5.6 kb could be detected in both normal HKc and HKc/HPV16 (Fig. 2); however, EGFR mRNA levels varied considerably among normal HKc strains and were markedly increased in all HKc/HPV16 lines, each compared with its individual normal HKc strain (Fig. 2). The same messages were detected in two HKc lines immortalized by infection with retroviral vectors expressing

either E6 and E7, or E7 alone. EGFR mRNA levels were similar in the two lines and approached those of a line containing full-length HPV16 DNA (Fig. 2). Southern blot analysis of DNA isolated from the HKc/HPV16 lines and their normal HKc strains of origin once again showed no evidence of amplification of the *EGFR* gene (data not shown).

Overall, these results demonstrated that the increase of EGFR mRNA levels observed after transfection with HPV16 DNA was a direct consequence of the presence and expression of HPV16 sequences in the cells, and not an artifact of long-term culturing. EGFR increases were detectable already in cells that had an extended life span but were not yet immortal. In addition, lines containing either E6/E7 or E7 alone showed similar EGFR levels. Therefore, we set out to investigate whether acute expression of single HPV16 oncoproteins was sufficient to increase the levels of EGFR mRNA in normal HKc. Individual normal HKc strains were infected with either the LXSN vector, or LXSN-16E6, LXSN-16E7, LXSN-16E5, or LXSN-6E6 (gift of Dr. Denise Galloway). After infection, cells were either incubated for 24 h and harvested for RNA extraction or selected with G418 for 4 days and allowed to recover for an additional 4 to 5 days before RNA extraction. Fig. 3 presents the results of two experiments, in which cells were first selected with G418 then allowed to recover and harvested for RNA extraction. In these cells, HPV16 E6 produced an increase in EGFR mRNA. In subsequent time-course experiments, we determined that the E6-induced increase in EGFR mRNA in normal HKc was detectable as early as 24 h and sustained at least up to 10 days after infection (data not shown). HPV16 E7 alone, HPV16 E5, or HPV6 E6 did not increase EGFR mRNA levels in normal HKc (data not shown). A summary of the results of the infection experiments we performed using individual normal HKc strains is presented in Table 1.

About 56% of the individual normal HKc strains we tested did not respond to E6 with an increase in EGFR (Table 1) despite the fact that E6 mRNA was expressed and p53 was degraded as expected in these cells, indicating that active E6 protein was produced (Fig. 4). EGFR mRNA and protein levels decrease dramatically as normal HKc approach senescence.[6] Therefore, we standardized the protocols for the establishment of primary HKc cultures, collected normal HKc from 1-day-old foreskins, and used only primary cultures for infection. Despite these precautions, we continued to detect marked interindividual variability in the expression levels of the EGFR mRNA and protein in normal HKc and in their response to E6. To assess whether the differences in EGFR levels we observed in cultured HKc were constitutive, we performed EGFR immunostaining of normal human foreskin tissue, using fresh tissue specimens processed in parallel from collection to staining (Fig. 5). These experiments confirmed that there is a marked interindividual variability in the EGFR expression levels in human skin. We observed that the normal HKc strains that responded to E6 expressed relatively low levels of EGFR mRNA, grew poorly in culture, and tolerated the infection protocol with difficulty. However, these cells responded to E6 with an increase in EGFR levels and robust proliferation; whereas normal HKc strains expressing relatively high basal EGFR levels grew well in culture but did not respond to E6 with increased proliferation and/or EGFR levels. The fact that only low EGFR expressors were susceptible to increasing EGFR levels in response to E6 prompted us to ask whether overexpression of the EGFR may have adverse effects on the proliferation and/or the life span of the cells. Therefore, we cloned the full-length human EGFR cDNA into the LXSN retroviral vector, produced a defective LXSN retrovirus able to efficiently transduce the EGFR into cells, and used this retrovirus to infect normal HKc. Infected cells were selected with G418, then their growth properties and life span were determined. Cells were counted at every passage and replated in equal numbers (350,000 cells/100-mm dish) to allow for an estimation of the PD in each culture. In the time interval in

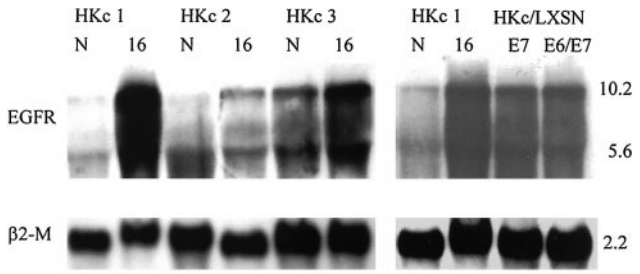

Fig. 2. Northern blot analysis of EGFR expression in normal HKc (*N*), HPV16-transfected HKc (16), or HKc immortalized with 16E7 or 16E6/E7 retroviral constructs. The total RNA from three different strains of HKc (*HKc 1*, *HKc 2*, and *HKc 3*) was isolated before (passage 1) and after (passages 5–10) transfection with linearized, gel-purified, full-length HPV16 DNA. RNA was also isolated from two immortalized lines established from a single normal HKc strain with LXSN-16E7 and LXSN-16E6/E7 recombinant retroviruses. EGFR mRNA levels were analyzed using a radiolabeled EGFR cDNA probe. The membranes were stripped and rehybridized with a β2-microglobulin (*β2-M*) probe as a loading control.

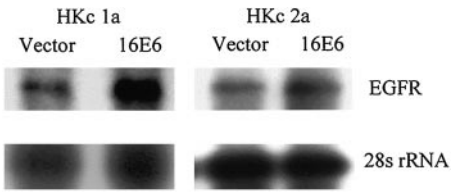

Fig. 3. RNase protection analysis of *EGFR* gene expression in HKc infected with LXSN-16 E6 recombinant retroviruses. Two different strains of HKc (*HKc 1a* and *HKc 2a*) were infected with 16E6 recombinant retroviruses (or vector control) and G418 selected for 4 days. Total RNA was isolated and the EGFR mRNA levels were analyzed by hybridization with an antisense RNA EGFR probe (probe IX; see "Materials and Methods"). An antisense 28 s rRNA riboprobe (Ambion) was used as a control.

Table 1 *Summary of results of retroviral infection experiments*

ORF	No. of individuals	No. of individuals with increased EGFR mRNA
6 E6	3	0
16 E6	18	8
16 E7	8	0
16 E5	5	0

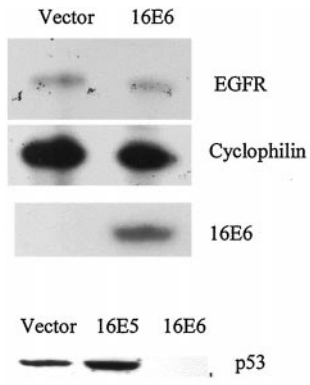

Fig. 4. Verification of 16E6 expression and activity in a normal HKc strain unresponsive to E6-mediated EGFR induction. EGFR mRNA levels were analyzed by RPA using antisense probes for EGFR and cyclophilin (as a control). HPV16 E6 expression was confirmed by Northern blot analysis using a PCR-generated E6-specific probe. Western blot analysis for p53 was performed to confirm 16 E6 activity in the infected cells.

[6] G. S. Akerman, E. Mourateva, and L. Pirisi, unpublished observation.

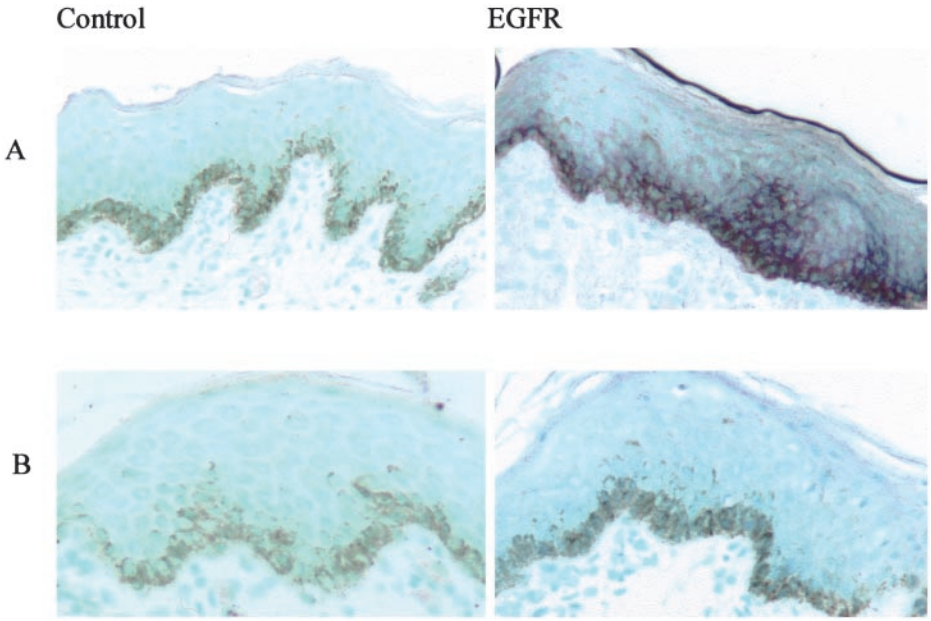

Fig. 5. Variability of EGFR expression in normal foreskin tissue. Foreskin tissue from two different individuals (*A* and *B*) was formalin-fixed, paraffin-embedded, and immunostained with an anti-EGFR antibody using the Vectastain Elite kit to compare EGFR levels. The nuclei were counterstained with methyl green. Control slides were incubated without primary antibody.

which vector LXSN-infected cells achieved ~3.5 PD, LXSN-EGFR-infected cells doubled <1.5 times. This experiment was performed using normal HKc derived from two different individuals with nearly identical results. When controls were nearly confluent, cells were trypsinized and replated, again in equal numbers. The morphology of the cultures at different passages after G418 selection is shown in Fig. 6; controls retained a nondifferentiated morphology and formed colonies of actively proliferating cells well after the EGFR-infected cells had ceased all proliferation. Therefore, acute expression of the EGFR is detrimental to cell growth and induces early senescence and/or apoptosis.

On the basis of these results, we asked whether the expression of E7, which by itself does not increase EGFR mRNA levels when acutely expressed in normal HKc, could allow for E6 induction of EGFR in normal HKc. Infection of normal HKc with both E6 and E7 LXSN viruses showed that acute expression of E6 and E7 together increases EGFR levels in normal HKc that were unresponsive to E6 alone (Fig. 7). This experiment was repeated using a second normal HKc strain with similar results. We also explored whether HKc infected with E7 alone and cultured past the senescence point of their controls had increased EGFR levels; EGFR levels increased in these cells as well, by passage 9 after infection (Fig. 7). Thus, the increase in EGFR levels we observed in HKc immortalized by E7 alone (Fig. 2) arises relatively early during E7-mediated transformation, although it is not directly elicited by acute expression of E7 alone.

DISCUSSION

We had previously shown that high EGFR expression is a constant characteristic of HPV16-transformed HKc. An additional increase in EGFR protein levels, without a concomitant increase in EGFR mRNA, occurs in HKc/GFI at the second stage of our multistep model for HPV16-mediated carcinogenesis *in vitro* (8) when cells acquire the ability to proliferate independently of EGF and BPE. In HKc/GFI,

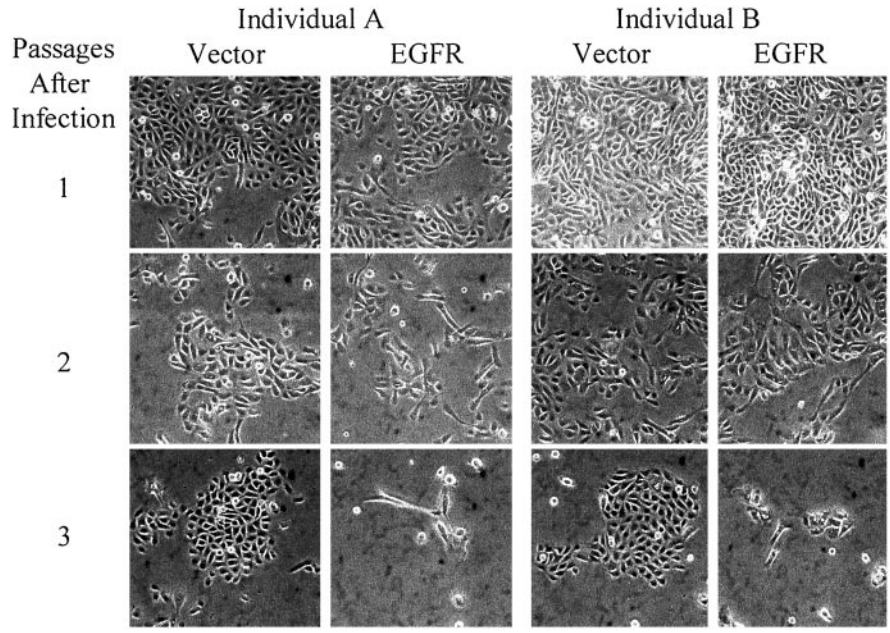

Fig. 6. Photomicrographs of two individual HKc strains infected with a recombinant EGFR retrovirus. HKc (*Individuals A* and *B*) were infected with the recombinant LXSN-EGFR retrovirus or the LXSN vector control. The cells were cultured as described in the text, and photographs were taken at three successive passages subsequent to infection to document changes in morphology and proliferation. ×100.

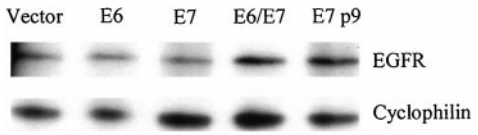

Fig. 7. Responsiveness of EGFR levels to E6 and/or E7 in HKc. Primary HKc were infected with LXSN-16E6, LXSN-16E7, LXSN-16E6, and -16E7 together, or with vector control recombinant retroviruses. Cells were selected in G418, and total RNA was extracted at passage 1 (*V, E6, E7,* and *E6/E7*) or passage 9 (*E7 p9*) after G418 selection. RPA was performed with an EGFR riboprobe (probe IX) to compare EGFR mRNA levels. Cyclophilin was used as an internal control.

increased levels of EGFR are necessary to support autonomous growth, because blocking anti-EGFR antibodies suppress clonal growth of HKc/GFI in the absence of EGF (8). These results point to a key role of the EGFR at the first two stages of HPV16-mediated transformation in our *in vitro* model system.

The data presented in this paper expand upon our initial observations, to demonstrate that acute expression of HPV16 E6 and E7 oncoproteins in normal HKc is accompanied by an increase in EGFR mRNA levels. E6 and E7 cooperate, each with a separate role, to produce this increase, which occurs as early as 24 h after infection of normal HKc. Acute expression of HPV16 E7 alone, HPV16 E5 alone, or HPV6 E6 does not increase EGFR mRNA levels.

In a subset of normal HKc strains, acute expression of HPV16 E6 alone produces an increase in EGFR levels independently of immortalization. The effect of E6 on EGFR levels was variable among different HKc strains, being detectable in ~44% of the normal HKc strains we analyzed. The bases of this variability in the responses to E6 have been intensely investigated in our laboratory. The demonstration of E6 mRNA expression and efficient p53 degradation in individual normal HKc strains that did not respond to E6 ruled out the possibility of a failure to express functional E6 in the nonresponsive cells, and it may indicate either that E6-induced EGFR overexpression is independent of p53 degradation or that p53 degradation is overcome by other mechanisms in the nonresponders. Responsiveness to E6 correlated with basal levels of EGFR expression: nonresponders had relatively high levels of EGFR expression, whereas the strains that responded to E6 with an EGFR increase expressed comparatively lower basal levels of EGFR mRNA and protein. It must be noted here that, in high- and low-expressors alike, the levels of EGFR decrease dramatically in normal HKc as the cells approach senescence. In addition, as they approach senescence, HKc are more difficult to transduce with retroviruses, given their limited proliferative potential, and become more difficult to transfect and refractory to immortalization by full-length HPV16 DNA. Therefore, we sought to ensure that the variability of EGFR levels we detected was constitutive and not linked to the age of the culture. For this reason, all experiments were conducted using primary cultures from 1-day-old skin specimens. An additional assurance that EGFR levels are constitutively different in individual HKc strains derives from the observation that EGFR staining varies dramatically among foreskin specimens derived from different donors and among normal HKc strains from different donors in culture. Therefore, we are confident that the difference in levels of EGFR expression that correlates with E6 responsiveness is an intrinsic property of the individual HKc strains, distinct from variations in EGFR mRNA levels associated with culture conditions or senescence.

The fact that E6 is unable to produce EGFR overexpression in cells that already express high levels of this receptor indicated to us that EGFR levels (and therefore, we assume, EGFR signaling) are tightly regulated in normal HKc, and that E6 alone is unable to overcome the mechanisms that prevent an excess of EGFR signaling. To confirm this hypothesis, we overexpressed the EGFR in normal HKc by infection with an LXSN-EGFR retrovirus and determined that EGFR overexpression was linked to a marked decrease in proliferation and to early senescence. These results are in agreement with observations made in other primary cell systems. For example, in normal human fibroblasts, "excessive" ras or EGFR signaling is conducive not to increased proliferation but to early senescence (Refs. 13 14 and references therein). Whether EGFR overexpression in normal HKc causes growth arrest, apoptosis, or early senescence is currently under investigation, however these results provide strong evidence that normal HKc tightly regulate EGFR levels/signaling.

These results show that HPV16-mediated immortalization is constantly associated with a disruption of the normal regulation of EGFR levels, strongly suggesting that EGFR increases do play a role in immortalization. Transfection with full-length HPV16 DNA invariably produces a robust increase in EGFR levels in normal HKc, and EGFR levels in HKc immortalized with E7 alone were as high as in those immortalized by LXSN/16E6E7; therefore, we suspected that E7 may disrupt such regulation mechanisms. This suspicion was confirmed by the observations that acute expression of E6 and E7 together increased EGFR levels in two normal HKc strains unresponsive to E6 alone, and that EGFR levels increased in cells with extended life span expressing E7 alone. Current studies in our laboratory are aimed at clarifying further the mechanisms by which E6 increases EGFR levels, the contribution of E7 to this pathway, and the functional role of these activities in immortalization.

These results, together with previous work of our laboratory and others, strongly support a role of EGFR increases at different stages of HPV16-mediated transformation of HKc and imply that high levels of EGFR are important in HPV16-mediated immortalization and in the *in vitro* progression of HPV16-immortalized human epithelial cells. Increases in EGFR levels are common in human cancer, including cervical cancer (15–26). Whereas in some cases the increase is a consequence of *EGFR* gene amplification, in stage I and II cervical cancer, the EGFR is more often overexpressed in the absence of gene amplification (23). In precancerous cervical lesions, both an increase in total expression levels and an abnormal distribution of EGFR within the differentiating epithelia has been demonstrated, with a loss of the preferential basal expression we also see in normal human foreskin (Fig. 5) and a more uniform distribution of EGFR staining throughout the entire epithelium (17). Most importantly, EGFR overexpression in cervical squamous cell carcinomas has been recently linked with a more aggressive clinical behavior and decreased disease-free survival (21, 23, 24). However, no correlation of high EGFR staining with the levels of expression of HPV oncoproteins has yet been made in cervical cancer specimens. Clues as to the possible mechanisms by which E6/E7 affect EGFR levels may be derived from a recent study of HeLa cells, where decreased expression of HPV18 E6/E7 obtained by the means of an antisense E6/E7 construct dramatically reduced both EGFR protein levels and proliferation (22). Interestingly, the same effect was elicited by overexpression of Rb (22) indicating that the effect of E7 on EGFR overexpression is mediated by Rb. However, in the same report, the reduction of EGFR levels caused by antisense E6/E7 or Rb occurred at the protein level with no changes in EGFR mRNA levels (22). This is quite different from what we observe with HPV16 E6/E7 in foreskin HKc. Therefore, additional studies are needed before conclusions reached using one experimental system can be applied to another. It will be of interest to explore whether overexpression of the EGFR correlates with the levels of expression of viral oncoproteins in cervical intraepithelial neoplasia, and whether these parameters can be used as biomarkers for risk of squamous intraepithelial lesion progression.

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