Keratinocyte Growth Factor Is a Bifunctional Regulator of HPV16 DNA-immortalized Cervical Epithelial Cells

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Abstract. Various factors are known to regulate cell growth and differentiation in epithelial-mesenchymal interactions. Keratinocyte growth factor (KGF), an epithelial-specific cytokine produced by dermal fibroblasts and other mesenchymal cells, appears to affect growth, migration, and differentiation in epithelial-mesenchymal interactions. We have previously shown that human embryonic skin fibroblasts induce anchorage-independent growth of HPV16 DNA-immortalized human uterine exocervical epithelial cells (HCE16/3 cell line) in cocultures of HCE16/3 cells and fibroblasts. Here we report that KGF may be a major factor influencing growth and behavior of HCE16/3 cells in the coculture system. KGF stimulated both DNA synthesis and proliferation of normal human cervical epithelial (HCE) cells and HCE16/3 cells and the increase was stronger in HCE16/3 cells than in HCE cells. SiHa cells, a cervical carcinoma cell line with integrated HPV16 DNA, did not respond to the KGF mitogen signal. KGF receptor (KGFR) studies suggested that the different responses to the KGF mitogen signal may be correlated with KGFR. In addition, KGF alone was able to induce anchorage-independent growth of HCE16/3 cells, suggesting a potential role for KGF in the transformation process of epithelial cells. However, the transcription of HPV16 early genes was suppressed by KGF in the immortalized HCE16/3 cells, and this appeared to be due to transcriptional repression rather than a posttranscriptional process according to nuclear run-on analysis. In contrast, viral gene expression was not affected by KGF in SiHa cells. Our results suggest that KGF is a bifunctional growth factor in the HPV-immortalized cells, a positive regulator of cell growth and negative regulator of HPV16 early gene expression.

The epithelial-mesenchymal interaction is a complex process. Stromal fibroblasts synthesize and release keratinocyte growth factor (KGF)[1] and scatter factor/hepatocyte growth factor (SF/HGF) that influence epithelial cell growth and migration (Panos et al., 1993; Sonnenberg et al., 1993). On the other hand, epithelial cells also produce and secrete interleukin-1 affecting the metabolic activity of fibroblasts (Goldyne et al., 1991).

KGF is made by stromal fibroblasts and belongs to the nine-member FGF family (Finch et al., 1989; Aaronson et al., 1991). In contrast to the other known members of the FGF family, KGF has a unique target cell specificity restricted to the epithelial cell type (Finch et al., 1989; Rubin et al., 1989), whereas acidic and basic FGF exert potent effects also on fibroblasts, endothelial cells, melanocytes and neurons (Basilico and Moscatelli, 1992). KGF is synthesized as a single polypeptide precursor that is cleaved upon secretion to liberate mature KGF (Finch et al., 1989). In vitro studies have shown that the stimulating effects of KGF on DNA synthesis in keratinocytes is 2- to 10-fold stronger than that of autocrine keratinocyte growth factors such as transforming growth factor alpha (TGF-α) and EGF (Rubin et al., 1989). Molecular cloning of the KGF receptor established that it is a membrane-spanning tyrosine kinase derived from an alternatively spliced form of the *bek* (FGFR-2) gene. This alternatively spliced transcript has been detected only in epithelial cells and the potent mitogenic activity on epithelial cells suggest that KGF is important in the physiological renewal of the epidermis. Guo et al. (1993) have shown that KGF has significant influence on the morphogenesis and organogenesis during development. Furthermore, Werner et al. (1992) recently reported strong induction of KGF expression in the dermis during wound healing, implicating that KGF-mediated effects may be important for the migration and proliferation of epidermal keratinocytes seen during wound healing.

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1. *Abbreviations used in this paper*: HCE, human cervical epithelial; HES, human embryonic skin; KDE, keratinocyte-dependent enhancer; KGF, keratinocyte growth factor; KGFR, KGF receptor; LCR, long control region; SF/HGF, scatter factor/hepatocyte growth factor.

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Little is known about the possible effects of KGF in epithelial carcinogenesis. Recent studies have shown that in the malignant progression, prostate epithelial cells cease to be responsive to KGF which can both support the growth of epithelial cells and induce them to a differentiated state (Marchese et al., 1990; Yan et al., 1993). The HCE16/3 cells that were used in the present study, are HPV16 DNA-immortalized human cervical epithelial cells, growing continuously and expressing early genes of HPV16 DNA, but fail to grow in soft agarose or in nude mice (Zheng et al., 1994). Their phenotype thus represents an early stage of human epithelial carcinogenesis in the multistep model of tumor development in vitro. We have now investigated the effect of KGF (*a*) on DNA synthesis and cell proliferation of different cervical cell lines; (*b*) on anchorage-independent growth of normal epithelial cells and immortalized epithelial cells; and (*c*) on HPV16 gene expression of HCE16/3 cells and the HPV16-positive SiHa cervical carcinoma cell line. We also studied specific binding of ^{125}I-KGF to its receptor in the different cervical cells. Our studies demonstrate that KGF strongly stimulates the growth of HCE16/3 cells and induces their anchorage-independent growth, but perhaps a more interesting and unexpected result is that KGF markedly inhibited the expression of HPV16 early genes in HCE16/3 cells.

Materials and Methods

Cell Cultures

The HCE16/3 cell line (Zheng et al., 1994) consists of HPV16 DNA-immortalized human uterine exocervical epithelial cells, which were cultured in a 1:1 (vol/vol) mixture of Dulbecco's modified Eagle's medium and Ham's Nutrient Mixture F12 (DME/F-12) supplemented with 2% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 400 ng/ml hydrocortisone, 10^{-10} M cholera toxin, and 5 ng/ml EGF in a 5% CO_2 incubator at 37°C. Characterization of HCE16/3 cells has been described in detail previously (Zheng et al., 1994). The cells were shown to be free of mycoplasm as determined by a hybridization test (Mycoplasm TC; Gen-Probe, San Diego, CA) and were used between passages 40 and 51 in the present experiments. Normal human cervical epithelial (HCE) cells were prepared in this laboratory as previously described (Zheng et al., 1994) and were maintained in the same medium as HCE16/3 cells. The culture medium was replaced three times per week. SiHa cells (HTB 35; obtained from American Type Culture Collection, Rockville, MD) derived from HPV16 DNA-positive cervical squamous carcinoma, were cultured in MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (10%-MEM). BALB/MK-2 cells (Weissman and Aaronson, 1983; a mouse epidermal keratinocyte line) were obtained from Dr. Jorma Keski-Oja (Department of Virology, University of Helsinki) and were cultured in medium with the following composition: 482 ml of Ca^{2+}-free 10%-MEM and 18 ml 10%-MEM). The medium was changed twice a week.

Keratinocyte Growth Factor

KGF was from Pepro Tech EC Ltd. (London, UK) and was reconstituted according to the manufacturer's recommendations.

DNA Synthesis Assay

BALB/MK-2, HCE (passage 2-3), HCE16/3 and SiHa cells were grown in 24-well 2-cm^2 plates (Greiner, Frickenhausen, Germany) for 3-4 d until the cultures were confluent. Cultures were serum-starved for 24 h (for HCE and HCE16/3 cells, the culture medium was used without the supplements). Then, KGF (0, 3, 10, 30, or 100 ng/ml) was added without change of medium; control cultures received the same volume of medium. 24 h later, the cells were labeled with 0.5 mCi/ml of [^{3}H]thymidine (26 Ci/mmol; Amersham, Aylesbury, UK) for 4 h. The labeling was terminated by washing the cells three times with cold PBS and fixing them with 95% methanol for 90 min at 4°C. The cells were then washed with cold PBS and extracted with 0.2 M NaOH for 30 min at 4°C. Liquid scintillation cocktail (Opti-Fluor®; Packard Instrument Company, Meriden, CT) was added to aliquots of the cell extract and the incorporated radioactivity was determined in a liquid scintillation counter. Each assay was carried out in triplicate.

Cell Proliferation Assay

BALB/MK-2, HCE (passage 2), HCE16/3 and SiHa cells were grown in 12-well cell culture plates in their respective media containing 2% serum (BALB/MK-2, HCE16/3, SiHa) or (HCE) 2% serum and insulin, transferrin, hydrocortisone, cholera toxin, and EGF as described above. After inoculation of 100,000 cells (BALB/MK- 2, HCE, HCE16/3) or 60,000 cells (SiHa) per dish different concentrations of KGF was added. Triplicate wells were counted after two and four (BALB/MK-2, HCE16/3, SiHa) or three and seven (HCE) days of culture. Growth medium was changed every second day.

Iodination of KGF

Iodogen tubes were prepared by placing 20 μl of choloroform containing 10 mg/ml Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Sigma Immunochemicals, St. Louis, MO) in the bottom of 3-ml glass tubes and then allowed to air-dry. Recombinant KGF was labeled by mixing 3 μg KGF/100 μl PBS and 50 μl 4 mM EDTA, pH 7.8, in the iodinating tube, adding 0.5 mCi ^{125}I/5 μl and incubating for 2-3 min at room temperature. The iodinated protein was isolated on a disposable column containing Sephadex G-25 (PD-10; Pharmacia, Uppsala, Sweden) pretreated with 50 ml Dulbecco's PBS containing 0.5% BSA and washed with 100 ml PBS before use. The specific activity of ^{125}I-KGF was 22,000 cpm/ng protein. ^{125}I-KGF was checked on 12% SDS-PAGE.

KGF Receptor Studies

The receptor binding assay was carried out as described by Bottaro et al. (1990), with small modifications. Briefly, confluent HCE cells (passage 3), HCE16/3 cells and SiHa cells were cultured in 24-well plates and serum-starved for 24 h (for HCE and HCE16/3 cells, the culture medium was used without the supplements). The cells were incubated with Hepes binding buffer (100 mM Hepes, 150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8.8 mM dextrose, and 0.1% BSA, pH 7.4) containing ^{125}I-KGF for 3 h with shaking at 4°C. The labeling was terminated by washing the cultures with cold PBS three times. The cells were then lysed with 0.5% SDS and the radioactivity was measured by a gamma counter. Each assay was carried out in duplicate.

Cross-linking of ^{125}I-KGF to receptor assay was also based on the previous procedure (Bottaro et al., 1990) with minor modifications. In short, the confluent HCE16/3 cells in 100-mm dishes were serum starved for 24 h and then 20 ng/ml ^{125}I-KGF was added in the presence or absence of 100-fold excess of unlabeled KGF. After 3-h incubation at 4°C, the plates were washed three times with cold PBS and disuccinimidyl suberate was added as described by Olwin and Hauschka (1986). After washing twice with cold PBS, the cells were scraped into 1 ml antiproteinase buffer (Hepes binding buffer containing 100 U/ml aprotinin and 1 mM PMSF), followed by centrifugation at 13,000 rpm at 4°C for 5 min. Membrane-associated proteins were extracted for 30 min in 35 μl of antiproteinase buffer containing 1% Triton X-100 at 4°C. After centrifugation, 35 μl Laemmli reducing sample buffer containing 100 mM DTT was added to the supernatant and the samples were boiled for 3 min. The samples were analyzed by 7.5% SDS-PAGE (Laemmli, 1970) and the dried gel was exposed to Kodak X-Omat film at -70°C with an intensifying screen.

Soft Agarose Assay

Cell culture 24-well plates were first coated with 0.5 ml of 0.64% sterile agarose (SeaPlaque agarose; FMC BioProducts, Rockland, ME) in 1.67-fold DME basal medium supplemented with 20% FCS. The agarose medium was solidified in a laminar hood. Next, 1.2-ml aliquots of 1.67-fold DME containing 16% FCS were prepared in 6-ml Falcon® tubes (2063; Becton Dickinson, Lincoln Park, NJ) and either 10^4 BALB/MK-2, HCE, or HCE16/3 cells with KGF (0, 50, or 100 ng/ml) were added. SiHa cells were used as a positive control. Then 0.8 ml of 1.0% sterile agarose, premelted and cooled to 45°C, was added to the tubes, mixed gently, and this mixture was layered on the precoated wells. The plates were cultured in a 5% CO_2 incubator at 37°C for 3 wk without further feeding. Colonies were scored after 14-18 d using an inverted microscope.

Northern Blot Analysis

HCE16/3 and SiHa cells were plated in 100-mm-diam dishes. To avoid the possibility that down-regulation of HPV gene expression by KGF might occur secondarily due to cessation of cell proliferation, subconfluent proliferating cultures were used for the experiments. The medium was replaced with fresh medium containing KGF. Northern blotting was carried out as previously described (Zheng et al., 1994). Briefly, total RNA was isolated by lysis of cells in guanidine thiocyanate and centrifugation through a CsCl cushion. 25 μg of RNA was fractionated on a 1.2% agarose gel containing 6.6% formaldehyde and transferred to membranes (Hybond™-C extra; Amersham) and the hybridization was performed as previously described. The HPV16 E7 probe was generated by polymerase chain reaction amplification of the E7 open reading frame on the template of HPV16 DNA (Zheng et al., 1994). The HPV 16 E6 probe was excised by PstI and EcoRI digestion from pEBF16/4 B plasmid (Fig. 1), kindly provided by Dr. Thomas Iftner (Institut für Klinische and Molekulare Virologie, Erlangen, Germany). This plasmid contained a fragment of HPV16 spanning nucleotides 7455–551 in the vector pGEM1 from Stratagene Inc. (La Jolla, CA). The plasmid was then transfected into *Escherichia coli* JM 109, amplified and purified with conventional methodology. Glyceraldehyde phosphate dehydrogenase probe was used to monitor the total RNA loading. Densitometric analysis was performed using a Rapid Electrophoresis Analyzer (Helena Laboratories, Beaumont, TX).

Nuclear Run-on Analysis

Subconfluent cells were grown in the presence or absence of 20 ng/ml of KGF for 24 h. After washing with cold PBS, cells were scraped and washed once with cold PBS. Isolation of nuclei from cells and nuclear run-on assay were performed basically using the method of Greenberg and Ziff (1984). Briefly, cells (2.5×10^7) were suspended in reticulocyte standard buffer: 10 mM Tris-HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT, and lysed by addition of Nonidet P-40 to a final concentration of 0.2% (vol/vol). The nuclear pellet was obtained by centrifugation at 500 *g* for 5 min at +4°C and then washed once in reticulocyte standard buffer. Nuclei were stored in 100 μl of 50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA at −70°C. For nuclear run-on assay, nuclei were thawed and mixed with 100 μl reaction buffer (10 mM Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$, 300 mM KCl, and 5 mM DTT, 0.5 mM each ATP, CTP, GTP), and 100 μCi [α-^{32}P]UTP (800 Ci/mmol), and incubated at 30°C for 30 min. The ^{32}P-labeled RNA was isolated by isopropanol-ammonium acetate precipitation. The HPV16 and β-actin cDNA inserts (1 μg/dot) were denatured by incubation with 0.1 M NaOH for 30 min at room temperature. The DNA was immobilized on nylon membranes (Pall Bio-Support Corp.; Glen Cove, NY) using a dot blot apparatus. Subsequently, the filters were dried and baked at 80°C for 2 h and prehybridized with the same solution as Northern blots. The filters were hybridized under the same conditions for 48 h in hybridization solution containing the elongated transcripts at 6.0×10^6 cpm/ml in each assay. After the hybridization, the filters were washed with 1× SSC and 0.1% SDS for 30 min at room temperature and 0.2× SSC and 0.1% SDS for 30 min at 60°C and treated with RNase in 2× SSC. Finally, the filters were exposed to Kodak AR X-Omat films at 70°C with an intensifying screen. The intensity of hybridization was measured by the scanning analyzer as Northern blots were.

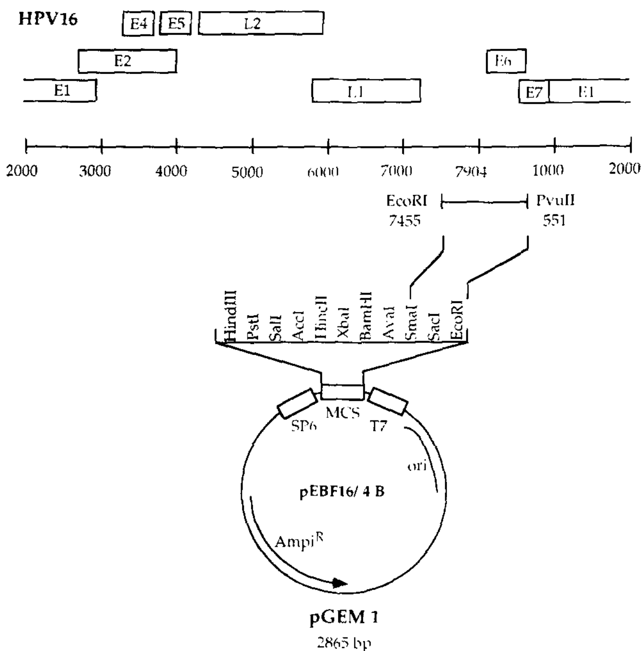

Figure 1. Molecular structure of the recombinant plasmid pEBF16/4 B, which contains a fragment of HPV16 spanning nucleotide 7455 (EcoRI) -551 (PvuII) in the vector pGEM1 from Stratagene. Since the blunt end of PvuII is joined with the blunt end of SmaI, this fragment is not recognized by PvuII and SmaI anymore; thus the used HPV16 E6 probe is between EcoRI and PstI. The genomic organization of the HPV16 DNA is shown on top of the figure (numbers indicate corresponding nucleotide sites).

Results

Stimulation of HCE16/3 cell Proliferation by KGF

The effect of KGF on cell growth was examined with a series of cell lines derived from human cervical epithelial cells. The primary objective was to determine whether KGF affected DNA synthesis and cell proliferation in a different manner in different human cervical epithelial cells. Effect of recombinant human KGF on [^{3}H]thymidine incorporation in HCE, HCE16/3, and SiHa cells, is shown in Fig. 2. BALB/MK-2 cell line was used as a control cell line known to respond to KGF (Rubin et al., 1989). HCE and HCE16/3 cells both responded to KGF stimulation. In HCE cells [^{3}H]thymidine incorporation increased 1.6-fold after addition of 10 ng KGF/ml and the uptake increased only slightly

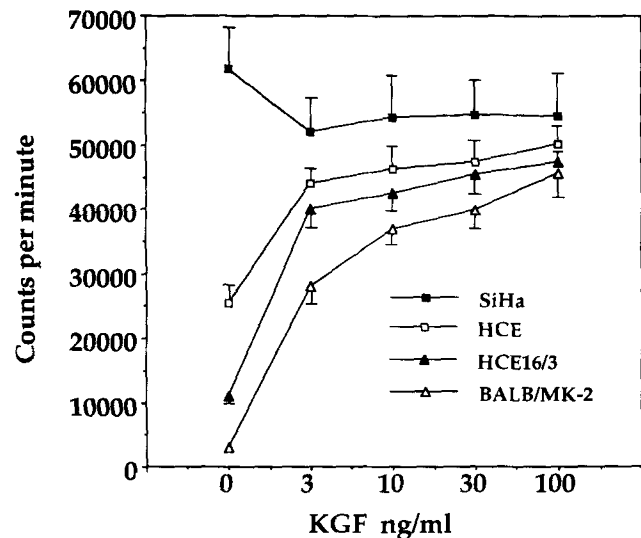

Figure 2. Effect of KGF on DNA synthesis as determined by incorporation of [^{3}H]thymidine in HCE16/13, HCE, SiHa, and BALB/MK-2 cell lines. Confluent cell cultures were serum-starved for 24 h and then exposed to 0, 3, 10, 30, or 100 ng/ml of KGF without change of medium. 24 h later, the cells were labeled for 4 h with [^{3}H]thymidine. The incorporated radioactivity was determined in a liquid scintillation counter. These data are the results (± SE) of a single experiment performed in triplicate. The experiment was repeated twice with similar results.

with higher concentrations of KGF (Fig. 2). [^{3}H]thymidine incorporation of HCE16/3 cells was stimulated 3.6-fold by 10 ng/ml KGF and it also increased slightly with increasing KGF concentrations. The observed increase in DNA synthesis was maximal, ~4.2-fold, at 30–100 ng/ml of the growth factor. In contrast to HCE and HCE16/3 cells, the [^{3}H]thymidine incorporation of SiHa cells was not affected by KGF.

The effect of KGF on cell proliferation correlated well with the above effects on thymidine incorporation (Fig. 3).

Presence of KGF Receptors on HPV16 DNA Immortalized Human Cervical Epithelial Cells

To investigate whether the different effects of KGF on DNA synthesis of HCE, HCE16/3 and SiHa cells were a result of differences in KGF receptors, we studied the binding of ^{125}I-KGF in these cell lines. Specific binding was determined by subtracting the activity found in samples incubated with 100-fold excess of unlabeled KGF from the activity bound to the cells in the absence of unlabeled KGF. All the experiments were carried out in the presence of 3 μg/ml heparin. HCE cells bound less KGF than HCE16/3 cells (Fig. 4). As expected SiHa cells exhibited no specific binding of ^{125}I-KGF to its receptor. Covalent affinity cross-linking of ^{125}I-KGF to its receptor on HCE16/3 cells was detected on SDS-PAGE. A single ^{125}I-labeled band was observed migrating at an apparent molecular mass of over 200 kD (Fig. 5). This complex, though somewhat larger than that described by others (Bottaro et al., 1990; Miki et al., 1992),

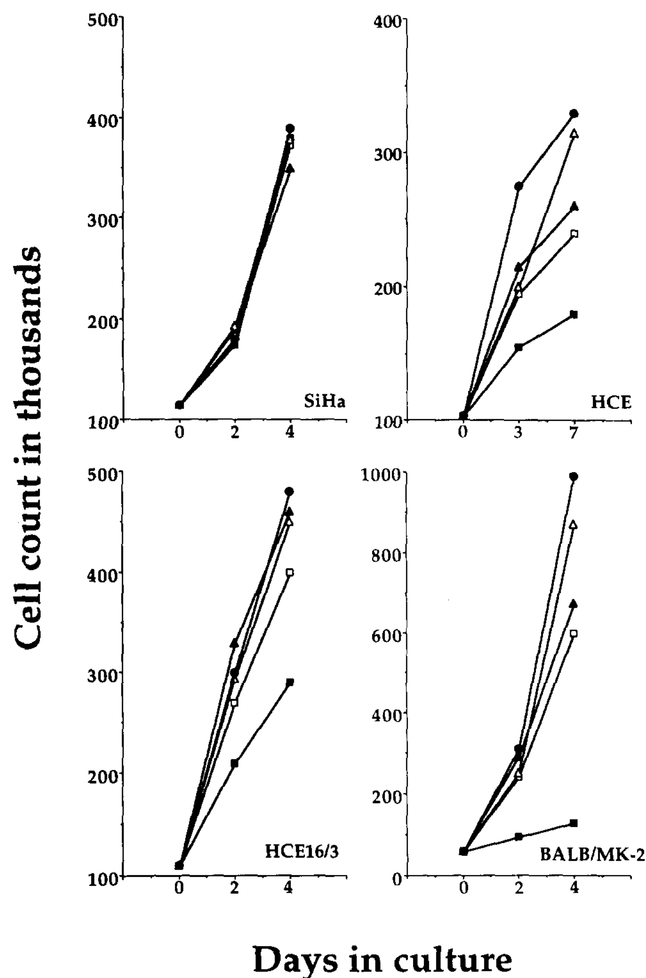

Figure 3. Effect of KGF on proliferation as determined by cell counting. Sparse cultures of SiHa, HCE, HCE16/3, and BALB/MK-2 cells were stimulated with increasing concentrations of KGF, and cells in triplicate wells were counted as indicated. The cervical carcinoma cells (SiHa) did not respond to KGF, whereas both normal and transformed cervical epithelial cells and mouse keratinocytes showed a dose-dependent increase in cell number with increasing concentrations of the growth factor. ■, 0 ng/ml; □, 1 ng/ml; ▲, 3 ng/ml; △, 10 ng/ml; ●, 30 ng/ml.

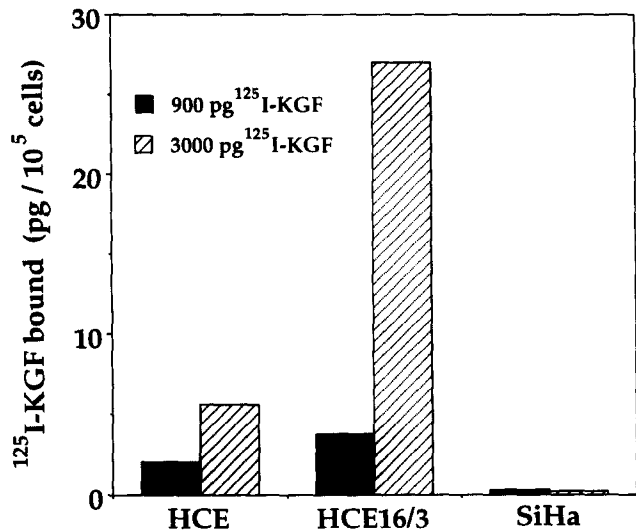

Figure 4. Binding of ^{125}I-KGF to KGF receptors in three human cervical cell lines. Confluent culture cells were cultured in 24-well 2-cm^2 plates and serum-starved for 24 h. The cells were incubated with Hepes binding buffer containing ^{125}I-KGF for 3 h at 4°C. The labeling was terminated by washing the cultures with cold PBS three times. The cells were then lysed with 0.5% SDS and the radioactivity was measured in a gamma counter. Background binding (subtracted) was determined in the presence of 100-fold excess of cold ligand and 3 μg/ml heparin. The data plotted are the mean of duplicate points.

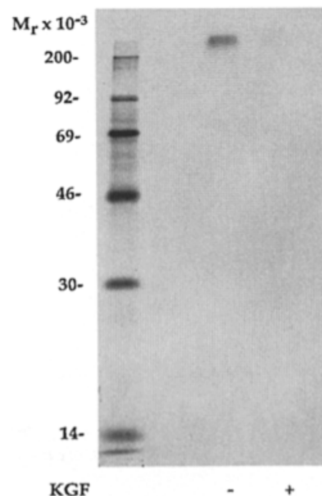

Figure 5. ^{125}I-KGF cross-linking to KGF receptor in HCE16/3 cells. Confluent HCE16/3 cells in 100-mm dishes were serum-starved for 24 h. 20 ng/ml ^{125}I-KGF were added in the presence or absence of 100-fold excess of unlabeled KGF. After a 3-h incubation at 4°C, the plates were washed three times with cold PBS and disuccinimidyl suberate was added. The samples were analyzed by 7.5% SDS-PAGE under reduced conditions and the dried gel was exposed for 3 d at −70°C. The second lane (labeled +) indicates the cross-linking performed in the presence of 100-fold excess of unlabeled KGF. The migration of molecular size standards (kD) is shown on the left.

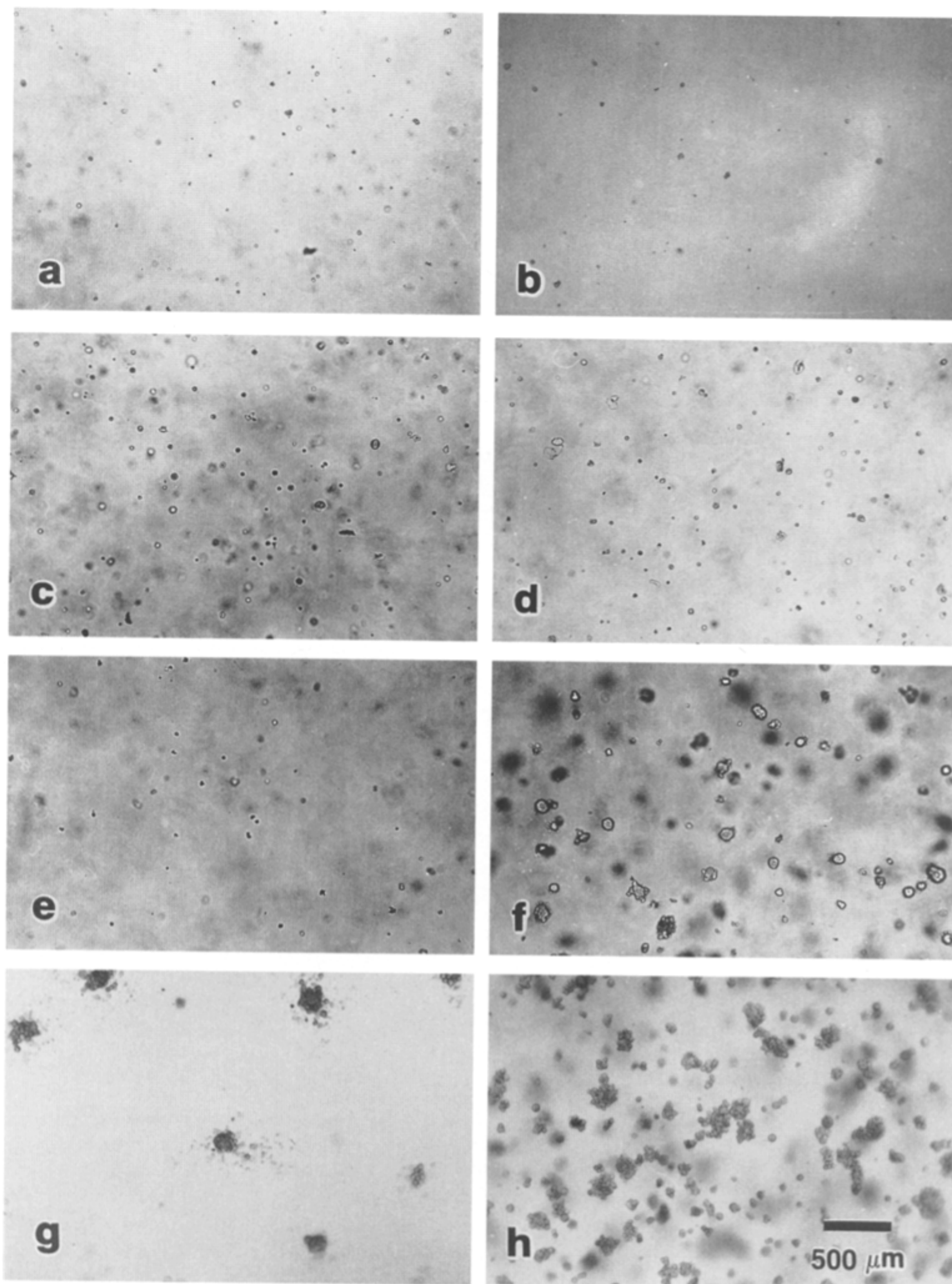

Figure 6. KGF-induced anchorage-independent growth of HCE16/3 cells. (*a*) 10^4 BALB/MK-2 cells; (*b*) 10^4 BALB/MK-2 cells with 50 ng/ml of KGF; (*c*) 10^4 normal HCE cells at passage 2; (*d*) 10^4 normal HCE cells with 50 ng/ml of KGF; (*e*) 10^4 HCE16/3 cells at passage 43; (*f*) 10^4 HCE16/3 cells with 50 ng/ml of KGF; (*g*) 10^4 HCE16/3 cells with 50 ng/ml of KGF and addition of 50 ng/ml of KGF every three days, total three times; (*h*) 10^4 SiHa cells without KGF as a positive control.

was abolished in the presence of 100-fold excess of unlabeled KGF, demonstrating specific binding of ^{125}I-KGF to its receptor.

KGF-induced Anchorage-independent Growth of HCE16/3 Cells

Neither normal cervical epithelial cells nor BALB/MK-2 cells, which respond to KGF stimulation by increased DNA synthesis, were able to grow in soft agarose in the absence or presence of KGF (50–100 ng/ml) while KGF induced anchorage-independent growth of the immortalized HCE16/3 cells at these concentrations (Fig. 6). Generally, HCE16/3 cells began to form visible colonies after 7–10 d. However, these colonies usually did not become very large and consisted of only 3–10 cells in most experiments. When cocultured with fibroblasts, HCE16/3 cells formed larger and more numerous colonies, nearly as effectively as SiHa cells, as previously reported (J. Zheng and A. Vaheri, manuscript submitted for publication). In separate experiments, we tested the dose-dependent effect of KGF (0, 10, 25, 50, and 100 ng/ml) on anchorage-independent growth of HCE16/3 cells. Although the maximal cellular response to KGF showed some variation from assay to assay, we found that 50 ng/ml of KGF was required to obtain a clearly positive result. The size and number of colonies induced by KGF was not significantly different between the concentrations 50 and 100 ng/ml. Generally each well contained ~230–600 colo-

nies (consisting of 5–15 cells) per 10^4 cells seeded. However, feeding additional KGF during the culture period increased the colony size (consisting of 10–100 cells), but did not significantly influence the colony number (Fig. 6 *g*). In separate experiments, neither bFGF (50 ng/ml; kindly provided by Dr. Andreas Sommer, Synergen, Inc., Boulder, CO) nor scatter factor (50–100 ng/ml; kindly provided by Drs. Water Birchmeier and Martin Sachs, MDC Max-Delbruck-Centrum, Berlin, Germany) alone could induce anchorage-independent growth of HCE16/3 cells or to have synergy with KGF in the induction of anchorage-independent growth of HCE16/3 cells (data not shown).

Inhibition of HPV16 Gene Expression of KGF

HPV16 early gene expression is thought to be primarily responsible for the continuous growth of HPV-immortalized cells in culture. HCE16/3 cells express three different mRNA species (1.7, 2.4, and 3.7 kb) hybridizing with the HPV16 E7 probe, and the 1.7- and 3.7-kb mRNAs are the major transcripts (Zheng et al., 1994). We determined 1.7- and 3.7-kb mRNA levels after KGF treatment using Northern blot analysis and densitometric scanning. Surprisingly, KGF induced downregulation of HPV mRNA levels in HCE16/3 cells (Fig. 7). A slight reduction (10%) of HPV16 E7 1.7-kb mRNA expression was observed after 8 h (20 ng/ml) and the inhibition became stronger with longer duration of KGF treatment (Fig. 7, *right part*). After 24–48 h the HPV16 early gene expression had markedly declined (~80% inhibition). The 3.7-kb mRNA appeared to be reduced more within 8–12 h (inhibition 40–50%), but the late reduction was similar to that of the 1.7-kb species. These different effects of KGF on the HPV16 1.7- and 3.7-kb mRNAs suggested that these RNAs are separately regulated. The effect of KGF on HPV16 E7 mRNA expression was reproducible in dose-dependence studies. Cultures on HCE16/3 cells were treated with KGF (10–100 ng/ml) for 24 h (Fig. 7, *left part*). In the presence of 10 ng/ml of KGF, the E7 1.7-kb mRNA expression was inhibited by ~70%, and in the presence of 100 ng/ml of the growth factor it was inhibited by ~80%. Rehybridization of the filter to cDNA of GAPDH, a housekeeping gene, demonstrated that similar amounts of RNA were present in each slot. Higher concentrations of KGF were not used, since they were considered unphysiological.

To exclude the possibility that the transcripts are initiated at a second promoter which is located in the E7 ORF (Böhm et al., 1993), the Northern blotting was repeated by hybridization with a HPV16 E6 probe, spanning the 5′ part of the E6 ORF (Böhm et al., 1993). The results demonstrated that HPV16 E6 mRNA was also inhibited by KGF treatment (Fig. 8), suggesting the inhibited transcripts are indeed initiated at the promoter p97 which is responsible for transcription of both early genes E6 and E7. Fig. 8 shows that E6 and E7 were cotranscribed in both HPV16-positive carcinoma cells (SiHa) and in the HPV16 DNA- immortalized human uterine exocervical epithelial cells, suggesting that continuous ex-

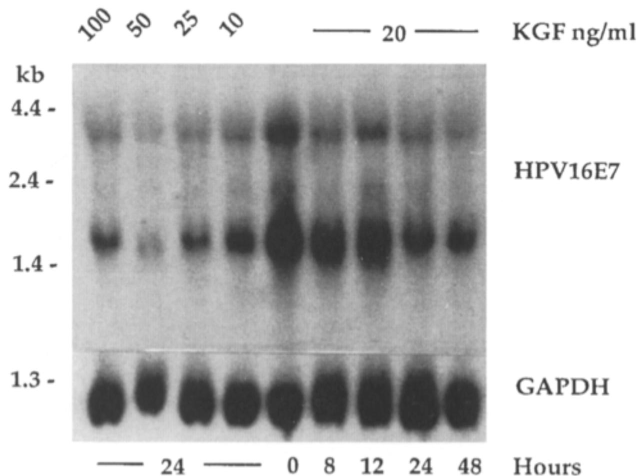

Figure 7. Northern blot analysis of the effect of KGF on HPV16 E7 mRNA expression in HCE16/3 cells. Proliferating HCE16/3 cells were exposed to KGF and cells were harvested at different times. Total RNA was isolated by lysis of cells in guanidine thiocyanate and centrifugation through a CsCl cushion. 25 μg of RNA were fractionated on a 1.2% agarose gel containing 6.6% formaldehyde, transferred to membranes and the hybridization was performed as previously described. After hybridization with the HPV16 E7 probe, the membrane was rehybridized with GAPDH probe used as a control for RNA loading. Inhibition of the 3.7- and 1.7-kb mRNA species is shown after different times and doses of KGF treatment.

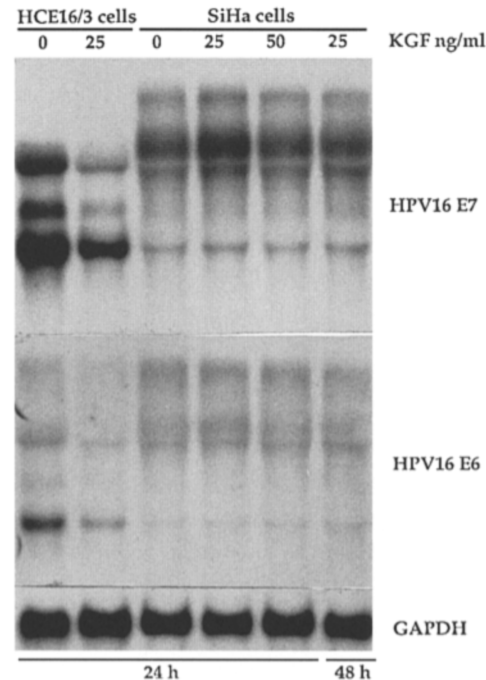

Figure 8. Northern blot analysis of the effect of KGF on HPV16 E7 and E6 mRNA expression in SiHa and HCE16/3 cells. Proliferating SiHa cells and HCE16/3 cells were exposed to 25-50 ng/ml of KGF for 24–48 h. Northern blot was carried out as described. HPV16 E7 expression in SiHa cells was not affected by KGF treatment; HCE16/3 cells were used as a control. After hybridization with HPV16 E7 and GAPDH, the filter was washed with solution containing 50% formamide and 1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate) at 70°C for 30 min, then 1× SSC and 0.1× SDS for 15 min at room temperature. The filter was rehybridized with HPV16 E6 probe.

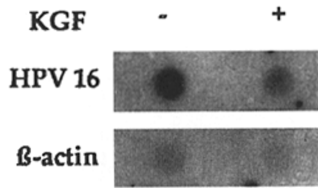

Figure 9. Transcriptional activity of HPV genes in HCE16/13 cells treated with KGF determined by nuclear run-on assay. Nuclei were isolated from proliferating cells treated with 20 ng/ml of KGF for 24 h. 1 μg of purified insert DNA from plasmids containing HPV16 and β-actin was immobilized on a membrane and hybridized to an equal amount of radioactivity (6.0 $\times$ 10^6 cpm) for 48 h at 42°C. The HPV16 hybridization signals from KGF-treated cells were clearly weaker than those from untreated cells whereas β-actin gene expression was not influenced.

pression of the viral early genes is required for the maintenance of the transformed phenotype. SiHa cells, which did not respond to KGF in their [^{3}H]thymidine incorporation, did not respond to KGF in their HPV16 gene expression (Fig. 8).

To discriminate whether down-regulation of HPV16 mRNA is due to a transcriptional repression or a posttranscriptional process, nuclear run-on experiments were carried out to quantitate the elongation rate of initiated transcripts. Proliferating HCE16/3 cells were incubated for 24 h in the presence or absence of 20 ng/ml of KGF, and nuclei were extracted for an in vitro transcription study in the presence of ^{32}P-labeled UTP. Transcription of the HPV16 mRNA was compared to that of β-actin. The HPV16 hybridization signals produced by the labeled run-on transcripts from KGF-treated cells were clearly weaker (30% suppression according to scanning values) than those from untreated cells, whereas β-actin gene transcripts were not influenced (Fig. 9). These data indicate that the KGF-mediated down-regulation of HPV16 mRNA, as observed by Northern blot analysis, is due to repression of transcription.

Discussion

KGF has been demonstrated to function as a paracrine growth regulator of epithelial structures both in vitro and in vivo (Aaronson et al., 1991; Werner et al., 1992; Guo et al., 1993; Yan et al., 1993), whereas its role in malignant transformation is less well studied. Here we show that KGF has profound effects on immortalized epithelial cells in vitro.

The HPV16 DNA-transfected cervical epithelial cell line (HCE16/3) used in our studies was sensitive to stimulation by KGF. Since more ^{125}I-KGF bound to HCE16/3 than to HCE cells, we assume that the receptor for KGF (KGFR) is upregulated in the immortalized cell line. Upregulation of other growth factors and/or their receptors has been found in certain human carcinomas (King et al., 1985; Kraus et al., 1989; McLeskey et al., 1994) and in HPV16-immortalized human keratinocytes (Sizemore and Rorke, 1993). On the other hand, the cervical cancer cell line, SiHa, exhibited a marked loss of responsiveness of KGF suggesting that it may lack KGF receptors or that all the receptors are occupied. The lack of complex formation even when a high concentration of labeled KGF was used, speaks for the absence of KGFR. This is consistent with the findings by Yan et al. (1993) using the prostate cancer model. In their model the transformation of epithelial cells lead to alternated splicing of the FGFR-2 (*bek*) gene and to a complete shift in the receptor specificity from the FGF-R2(IIIb) isoform displaying high affinity for KGF to the FGF-R2(IIIc) isoform not recognizing KGF but having high affinity for the FGF-2 member of the FGF ligand family. This shift resulted in an autocrine mode of growth stimulation. Thus, epithelial cells are able to alter their response to the mitogenic signal by KGF when they are in the process of malignant transformation.

The different responses to KGF suggest that HCE16/3 cells have retained a more normal epithelial phenotype than SiHa cells. The existence of wide differences in the biological characteristics between immortalized nontumorigenic and tumorigenic cells has also been observed in our previous work, where HCE16/3 cells failed to grow in soft agarose and were not tumorigenic in nude mice (Zheng et al., 1994). According to clinical observations there is a long course from premalignant cervical epithelial lesions to fully malignant invasive cells. The behavior of HCE16/3 cells in three-dimensional cell cultures bears a close resemblance to the early stages of cervical intraepithelial neoplasia (CIN) (Zheng et al., 1994), and the differences in regulation by KGF may indicate that at these CIN stages the cells are still controlled by their immediate environment. Carcinoma cells have largely escaped from growth control in vivo, they are more autonomous and no longer dependent on the availability of external growth factors.

The finding that KGF induced anchorage-independent growth of HCE16/3 cells is not surprising since human embryonic skin (HES) fibroblasts can induce it in HES-HCE16/3 cocultures (J. Zheng and A. Vaheri, manuscript submitted for publication), and KGF is an epithelial cell-specific growth factor produced by fibroblasts (Rubin et al., 1989). However, induction of anchorage-independent growth by KGF is not as efficient as that induced by HES fibroblasts, indicating that the effect may only partly be replaced by KGF and that also other factors produced by fibroblasts may be involved in our coculture system. Monoclonal neutralizing anti-KGF antibodies (10 μg/ml; kindly provided by Dr. J. S. Rubin, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD) did not fully block the biological effect of cocultured fibroblasts (data not shown), which is consistent with the above observations.

The mechanism of KGF-stimulated anchorage-independent growth of HCE16/3 cells is unclear. TGFs were the first recognized factors able to induce nontransformed cells to behave as tumor cells in vitro. Based on our results, we conclude that KGF shares certain functions with TGFs in conferring a transformed phenotype to nontransformed immortalized cells. However, it remains to be determined whether the soft agarose growth of immortalized cells induced by KGF is a typical anchorage-independent growth closely associated with the cell's tumorigenic capacity. Based on the knowledge of the involvement of a number of growth factor receptors in malignant progression, it will be important to further investigate the role of KGF and KGFR in human epithelial tumors.

It is not clear whether the down-regulation of HPV16 E7 gene expression by KGF was a direct effect or was mediated by modulating the levels of *trans*-acting factors that control the activity of e.g., the p97 promoter via complex host intra-

cellular control mechanisms (zur Hausen, 1989). Papillomavirus transcription is regulated by several distinct enhancers located within a 5' long control region (LCR), which responds to glucocorticoids, keratinocyte-specific factors and autoregulation by products of the HPV E2 gene (Sousa et al., 1990). The keratinocyte-dependent enhancer (KDE) is likely to contribute to the epithelial cell tropism of HPV, and mutational studies have shown that this site is required for full enhancement of virus gene expression (Chong et al., 1990). Because the target cells of KGF are epithelial, it is reasonable to propose that HPV mRNA inhibition may be associated with KDE. We speculate that KGF inhibits HPV16 early gene expression through interference with functions of KDE. KGF could directly interfere with KDE or indirectly through interference with cellular factors in the immortalized keratinocytes. The enhancer domains of LCR also contain binding sites for various cellular factors and although their role is not yet defined, it seems likely that they influence the transcription of viruses. The previous reports have shown that the expression of HPV16 genes is differentially controlled by cellular factors (Yasumoto et al., 1991). The relationship between KGF and these cellular factors remains to be clarified.

Another possible mechanism of inhibition of HPV16 early gene expression is similar to that by TGF-β1 which has been shown to down-regulate HPV16 early gene expression in HPV16-immortalized human genital epithelial cells by interference with a transcription factor (Woodworth et al., 1990). Although TGF-β inhibits proliferation of epithelial cells through the G1 phase of the cell cycle but not during the S phase (Pietenpol et al., 1990), the TGF-β effect on transformed cells varies significantly, depending on the cell line (Fynan and Reiss, 1993). Generally, transformed cells have a decreased sensitivity to the antiproliferative effect of TGF-β and become more resistant to TGF-β in malignant progression. One possibility is that TGF-β is an autocrine factor in HCE16/3 cells and is stimulated by KGF treatment and that TGF-β is responsible for the down-regulation of HPV early gene expression (and perhaps also for the anchorage-independent growth of these cells).

The observed reduction of HPV16 mRNA levels prompted us to more closely define the KGF effect on HPV16 DNA transcription. From nuclear run-on experiments we conclude that the suppression of HPV16 gene expression by KGF is regulated at the levels of initiation of transcription rather than posttranscriptionally. The finding that HCE16/3 cells show selective down-regulation of viral RNA provides a useful experimental system to study viral gene expression. It remains to be determined whether the inhibition of HPV mRNA expression by KGF is reversible or irreversible or whether the effect of KGF on HPV expression varies significantly in different cell lines like that of TGF-β (Fynan and Reiss, 1993).

The biological significance of KGF to down-regulate HPV early gene expression is not clear. Although it has been established that HPV DNA expression is required for cell transformation and for the maintenance of the transformed state, these requirements are not absolute. The precise role of E6 and E7 in these processes is still a matter of debate, and the relationship between cell growth and viral gene expression remains controversial. Woodworth et al. (1990) examined whether cell growth affected viral gene expression. They found that HPV RNA expression was similar in three conditions, in rapidly proliferating cells, when cultures were maintained in growth factor-deficient medium, and at confluent density. This meant that HPV gene expression was not necessarily inhibited by growth arrest. Agarwal et al. (1994) found that retinoid-dependent changes in HPV16-immortalized cervical cell proliferation do not always correlate with changes in E6/E7 transcript levels. In contrast, inhibition of the growth rate was found to down-regulate HPV E6 and E7 genes in C4-1 cervical carcinoma cells (von Knebel-Doeberitz, 1988) and the downregulation of HPV18 mRNA correlated directly with cessation of cellular growth (Rösl et al., 1988). Our results are consistent with those of Woodworth and Agarwal, showing the viral mRNA and cellular growth of HCE16/3 cells are probably controlled by different genes; thus viral mRNA does not directly modulate cell growth in our model.

Although it is clear the some members of the FGF family are oncogenic (Basilico and Moscatelli, 1992), so far nothing is known about the oncogenic potential of KGF. Guo et al. (1993) found that certain regions of the hyperthickened skin in old KGF-expressing transgenic animals showed features which were similar to the histopathological findings observed during the early stages of papilloma formation. Since KGF is a more potent mitogen for epithelial cells than EGF or TGF-α, it is reasonable to hypothesize that KGF may play a role in epithelial carcinogenesis.

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