Immortalization of Normal Human Bronchial Epithelial Cells by Human Papillomviruses 16 or 18

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ABSTRACT

Human papillomaviruses (HPV) are associated with papillomatosis of the larynx, trachea, and bronchi in decreasing order of frequency, and these papillomatosis lesions may become malignant. When the patients are not selected for a history of papillomatosis, the frequency of HPV in bronchogenic carcinoma tissue is 1–5%. In order to develop a model for investigating the role of HPV in human bronchogenic carcinogenesis, normal human bronchial epithelial cells were transfected with cloned full-length HPV16 or HPV18. Two HPV18-transformed cell lines (BEP1 and BEP2) and one HPV16-transformed cell line (BEP3) were established. These nontumorigenic epithelial cell lines have: (a) attained over 100 population doublings in vitro; (b) mutually exclusive human marker chromosomes; (c) HPV DNA in forms that are consistent with chromosomal integration by Southern analysis; (d) HPV E6, E7, and E6*-mRNA transcripts by Northern and reverse transcriptase-polymerase chain reaction analysis; and (e) diminished confluence-induced squamous differentiation. These cell lines should be useful for studying mechanisms involved in proliferation, differentiation, and neoplastic transformation of human bronchial epithelial cells.

INTRODUCTION

Bronchogenic carcinoma is the most common cause of cancer death among men and may now exceed breast cancer as the most common cause of cancer death among women in the United States (1). While it is clear that the major cause of bronchogenic carcinoma is cigarette smoking, other cofactors are involved (2).

One risk factor in a subgroup of bronchogenic carcinoma patients may be infection with HPV. There is strong evidence for the role of HPV as a human carcinogen in anogenital epithelial carcinogenesis (3) and in the hereditary condition, epidermodysplasia verruciformis (4). In addition, respiratory airway papillomatosis is now known to be caused by infection with HPV (5) and is associated with an increased risk for malignancy of the affected epithelial tissue (6, 7). There is evidence from case histories that in those with respiratory papillomatosis, therapeutic irradiation is associated with an increased risk for malignant transformation (8). In several epidemiological surveys of bronchogenic carcinoma occurring in the absence of papillomatosis, HPV DNA has been identified by in situ hybridization or filter hybridization in 1–18% of cases (9–11). Similar studies, using the polymerase chain reaction amplification technique (12) and primers that identify all known HPV strains (13), should provide a more accurate evaluation of the association of HPV with bronchogenic carcinomas occurring in the absence of papillomatosis.

The potential for HPV to alter regulation of proliferation has been confirmed by in vitro transfection of cervical, epidermal, and mammary epithelial cells, resulting in their "immortalization" (14–17).

The mechanisms by which HPV transform cells to continuously growing cell lines are currently being investigated very actively. The HPV genes responsible for immortalization are now known to be E6 and E7. While two studies have reported that for immortalization of human keratinocytes both E6 and E7 must be present (16, 18), when transfected alone, E7 induces hyperproliferation, but the cells eventually senesce; in contrast, E6 alone exhibits no detectable mitogenic activity (16). In addition, E7 alone will morphologically transform murine NIH3T3 cells (19).

The strains of HPV most closely associated with carcinoma of the cervix are HPV16, HPV18, HPV31, HPV33, and HPV35 (20). An interesting property of these strains that is not associated with other strains is signals for alternative splicing within the gene coding for the E6 protein (21–23). It has been determined that while both E6 and E7 are necessary for HPV transformation of epithelial cells to continuously growing cell lines, neither E6, E6*, nor E7 alone nor the combination of E6* and E7 without E6 is capable of transformation. Although the significance of the alternative splicing of E6 is not yet clear, the E6*/E7 transcript may serve as a better template for translation of E7 (24).

We report here the immortalization of NHBE cells by HPV16 or HPV18 and describe the resultant cell lines.

MATERIALS AND METHODS

Cell Culture. NHBE cells were cultured as outgrowths from human bronchial explants according to previously published methods (25). For clonal isolation, culture vessels containing well-separated colonies were rinsed twice with Hank’s buffered salt solution. Colonies were rinsed with stopcock grease which had been autoclaved, or circled with needle scratches in order to isolate them during dissociation and to ensure removal of single colonies. For Mycoplasma testing, cultures were incubated for 2 weeks in antibiotic-free medium prior to testing. Cultures were tested for microbiological assay (26) every 3–5 months, and were negative.

The number of population doublings occurring between passages was determined by assuming a 50% attachment efficiency, and then dividing the log of the number of cells harvested by the log of 2 and subtracting the log of the number of cells plated divided by the log of 2.

Transfection

Lipofectin Transfection Protocol. The plasmids containing HPV16 (cloned at the BamHI site into pUC18) or HPV18 (cloned at the EcoRI site into pBR322) originally isolated by zur Hausen (20, 27) were kindly provided by P. Howley. Plasmid DNA was expanded and extracted by the Triton-lysozyme method (28). Lipofectin (Life Sciences-BRL, Rockville, MD) was used according to the instructions of the supplier. For each sample of DNA to be transfected, 100 µl of lipofectin was placed in a polystyrene tube. Four ml of LHC-8 medium (25) were added. Fifty µg of DNA (20 µg for HPV16) were added to a second tube containing 4 ml of medium. The two tubes were then mixed and added to dishes of cells. There were five dishes receiving lipofectin and...
the following: (a) sham; (b) pBR322 DNA (50 μg); (c) HPV16 DNA (20 μg; (d) HPV18 DNA (50 μg); (e) HPV18 DNA (50 μg). After incubation overnight at 37°C in an atmosphere with 3.5% CO₂, the medium with lipofectin was removed, cells were rinsed once with Hank’s buffered salt solution, and the cells were incubated in LHC-8. For early passages, cells were passed 1:2 every week. With increased robustness in later passages, cells were routinely passed 1:10 every 2 weeks.

Keratin Expression by Immunohistochemistry. Glass multiwell slides (Nunc, Inc., Naperville, IL) were inoculated with 10,000 cells/cm². After 24 h of proliferation at 37°C and 5% CO₂, the cells were fixed in acetone at −20°C for 10 min and stored at −20°C. Keratin expression was detected using a murine monoclonal antibody recognizing type 18 cytotekatin (antibody CK5; ICN Immunobiologicals, Lisle, IL). The primary antibody was incubated overnight at 4°C and was subsequently detected by a biotinylated secondary antibody and an avidin-biotin peroxidase system (Vector Laboratories, Inc., Burlingame, CA).

Cell Line Identification. Karyotype and isozyme analysis were carried out according to previously described methods (29). Molecular genetic fingerprint analysis was conducted by using three probes, the 33.6 and 33.15 multilocus probes, and the combination of the MS1, MS31, MS43, and g3 single-locus probes, according to previously published methods (30) with 5–10 μg DNA samples.

DNA and RNA Isolation. DNA was prepared from frozen tissue by phenol/chloroform extraction and from cell lines by either phenol/chloroform extraction or cesium chloride gradient centrifugation (32). RNA was stored as an ethanol precipitate at −70°C.

Southern Analysis. Evaluation for presence of HPV DNA sequences was by Southern analysis (33). DNA samples were digested with the indicated restriction endonuclease enzymes in buffers recommended by the manufacturers for 14–15 h in a volume of 150 μl at 37°C. They were then ethanol precipitated, taken up in 20 μl of sample buffer, heated to 65°C for 10 min, loaded on 1% agarose gels, and electrophoresed for 14–16 h at 20 V in 40 mm Tris/20 mm acetate/1 mM EDTA buffer. The DNA was dissociated and transferred to Nytran (Schleicher & Schuell, Keene, NH) or GeneScreen Plus (NEN/DuPont, Boston, MA) membranes by vacuum transfer, using a Vacugene vacuum blotting unit according to manufacturers’ instructions. Following transfer, the Nytran membranes were baked at 80°C in a vacuum oven for 2 h. Probes were labeled either by random primer (34) or nick-translation (35) methods.

Slot Blot Analysis. The number of copies of HPV per cell of each cell line was determined by comparing DNA samples from BEP1 and BEP2 cell lines to samples from the cervical carcinoma cell line C4-1 which is known to contain a single copy of HPV18 DNA (36), and comparing DNA samples from BEP3 to those from SiHa which contains a single copy of HPV16 (37). DNA (4 μg) was denatured in 0.25 M NaOH for 10 min at room temperature, and was then 2-fold serially diluted in 0.125 M NaOH/0.125 M NaCl 7 times. The samples were applied to Nytran or GeneScreen Plus membranes by using a Schleicher & Schuell minifold II Slot-Blotter manifold. Hybridization with the indicated probes was as described for hybridization of Southern and Northern blots.

Northern Analysis. The RNA (20 μg/sample) was electrophoresed in a formaldehyde-agarose (1%) gel which was prepared according to previously described methods (38). Bacterial rRNA (23S and 16S) (Pharmacia, Inc., Piscataway, NJ) was used in addition to endogenous (28S and 18S) RNA as size markers. The RNA was electrophoresed for 3–4 h at 200 V. RNA was transferred to a Nytran or GeneScreen Plus membrane using vacuum transfer. Hybridization with 32P-labeled DNA probes was carried out as with Southern hybridizations described above.

Reverse Transcriptase Conditions. RNA, either extracted from cells or in sonicates of transformed cells was reverse transcribed at 42°C for 30 min in a total volume of 5 μl containing 50 mM Tris-HCl, pH 8.3, 7 mM KCl, 50 mM MgCl₂, 170 μg/ml bovine serum albumin, 1 mM dATP, dTTP, dCTP, dGTP, 0.1% Triton X-100, 1 mM dithiothreitol, 10 units Rnasin, 10 mM 2-mercaptoethanol, 0.2 μg of each primer and 7 units avian myeloblastosis virus reverse transcriptase.

Polymerase Chain Reaction Conditions. Complementary DNA obtained by reverse transcription was amplified in a total volume of 8.5 μl containing 50 mM Tris-HCl, pH 8.3, 7 mM KCl, 50 mM MgCl₂, 170 μg/ml bovine serum albumin, 1 mM each dATP, dTTP, dCTP, dGTP, 0.4 μg of each primers and 1 unit Taq polymerase. Samples were subjected to 35 cycles of amplification.

For amplification of HPV18, the following primers were used. L18: AGT GAA TTC TTC GTC ACT GCA AGA CA; R18: AGT GAA TTC GGC GCC TTA ATT GCT CGT GAC AT. A map of the amplified region and the spliced sequences within it is provided in Fig. 8 with polymerase chain reaction primers L18 and R18, 544-base pair DNA complementary to spliced and/or 362-base pair DNA complementary to spliced RNA were amplified. The primers for amplification of the E6/E7 region of HPV16 and Cu,Zn-SOD are as follows. HPV16, L16: AGT GAA TTC AGT ACT GCA AGC AAC AGT TAC TG; R16: AGT GAA TTC AAC GTT GTC TCT GGT TGC AAA TC; Cu,Zn-SOD LSOD: AAG TCC TCA CTT TAA TCC TC; RSOD: ATG ATG CAA TGG TCT CCT GA. Control lanes without template DNA were included to rule out contamination. C41 is a cervical carcinoma line known to contain a single copy of HPV18.

Tumorigenicity Assay. Cells were dissociated, washed with Hanks' buffered salt solution, and taken up in polyvinyl pyrrolidone at a concentration of 5 × 10⁵ cells/ml. Five × 10⁶ cells (in 0.1 ml) were injected i.c. into each of 10 athymic nude mice (irradiation the previous day with 350 rads) for each cell line.

RESULTS

Early Changes following Transfection. Immediately following the lipofectin treatment cells in all dishes appeared morphologically normal. After 24 h of incubation the HPV16- and HPV18-transformed cells appeared to have more mitotic figures per high powered field than the controls. During subsequent incubation, the cells transfected with HPV16 or HPV18 grew much more rapidly than the controls. Cells that were only treated with lipofectin but no DNA, and cells that were treated with lipofectin and pBR322 DNA continued to grow well until the third passage. They then ceased dividing, acquired a squamous morphology, and after several weeks, sloughed off the surface. In contrast, while most of the cells that had been transfected with HPV18 or HPV16 ceased dividing following the third passage, in each T150 flask, about 50 colonies of rapidly dividing cells became apparent. In the case of HPV18-transfected cells, these colonies from two original dishes were passed in separate mass cultures and were designated lines BEP1 and BEP2. From passage 4 to passage 8 the BEP1 and BEP2 culture appeared heterogeneous with colonies of small rapidly proliferating cells and a large fraction of the culture consisting of large, squamous differentiated cells (Fig. 1A). During this time the cultures were unable to attain confluence. Between the eighth and ninth passage, these lines became more homogeneous, entirely composed of small, rapidly proliferating cells (Fig. 1B), and acquired the ability to become confluent and maintain in a confluent nondifferentiated condition for more than 3 months. In the case of HPV16-transfected cells, 9 single colonies were isolated and expanded following the third passage. Five of the colonies have survived, and of the remaining isolates, BEP3D has been maintained in continuous culture. The HPV16-transformed cell line also underwent a change in morphological appearance and differentiation properties with increasing passage (Fig. 1, C and D).

Population Doublings and Tumorigenicity. While the untransfected cells and those transfected with plasmid alone ceased
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Fig. 1. Morphology of early and late passage HPV16- or HPV18-transformed bronchial epithelial cells. A and B, HPV18-transformed, population doubling 20 and 70, respectively. C and D, HPV16-transformed, population doubling 20 and 30, respectively.

Fig. 2. Population doubling record of HPV16- or HPV18-transformed bronchial epithelial cells. Dividing after 3 passages, or about 10 population doublings, those transfected with either HPV16 or HPV18 have continued to proliferate (Fig. 2) and now have achieved over 120 population doublings. The resulting lines BEP1, BEP2, and BEP3 were injected into athymic nude mice at approximately passages 6 and 18 and are not tumorigenic after an observation period of 12 months. While lines BEP1 and BEP2 have continued to proliferate at approximately 2–3 population doublings per week following passage 4, BEP3 clonal isolates proliferated at a much slower rate for approximately 30 population doublings. Presently, BEP3D divides at a rate somewhat slower than that of lines BEP1 and BEP2 (Fig. 2).

Immunocytochemical, Cytogenetic, Isozyme, and DNA Fingerprint Analyses. Immunocytochemical analysis of keratin type 18 expression in the BEP1, BEP2, and BEP3 cell lines demonstrated cytoplasmic staining in all cells of all three lines (data not shown).

BEP1 (passes 8 and 18), BEP2 (passes 6 and 17), and BEP3 (passage 14) were subjected to cytogenetic (Tables 1 and 2) and isozyme analysis. The BEP3 sample (BEP3D) and the late passage samples of BEP1 (BEP1B) and BEP2 (BEP2D) were clonal, whereas the early passage samples of BEP1 and
BEP2 evaluated were from mass culture. Results of isozyme analyses (lactate dehydrogenase, human; G6PD, B; PGM1, 1; PGM3, 1–2; ESD, 1; Me-2, 0; AK-1, 1; and GLO-1, 2) were consistent with a single human donor origin for all three lines. Cytogenetic analysis revealed aneuploidy of all three lines. Each line had characteristic marker chromosomes (Table 1). In the lines that were evaluated at more than one passage (BEP1 and BEP2), stable marker chromosomes were identified. In passage 8 cells from the BEP1 cell line isochromosome formation of the q arm of chromosome 3 was consistently present, an abnormality in the q arm of chromosome 7 was observed in a majority of the karyotypes prepared, and alterations in chromosome 11 and the q arm of chromosome 5 were observed in some karyotypes. Most of these cells had chromosome counts in the triploid range. In the passage 18 clonal isolate BEP1B, again most of the cells had chromosome counts in the triploid range. The distribution of markers was different in that while the del(7)(ql2q22) marker was present in only 1 of 10 karyotypes at passage 8, it was present in 5 of 7 karyotypes in the passage 18 clonal isolate. In addition, two new markers involving 3q (3qter>3p14::12q13>12qter and del(3)(p12::) were observed in a combined total of 5 of 7 karyotypes. This suggests that either a particular clone outgrew the others that were present at passage 8, or that cells with this karyotype became predominant as a result of the cloning effort. It will be of interest to determine whether these particular markers are conferring important phenotypic properties associated with immortalization.

Our study of BEP2 revealed that there was a stable monosity of normal chromosomes 12 and 13, and at least parts of these missing chromosomes were incorporated into a stable marker (Table 1). The rest of the karyotype in the BEP2D clonal isolate was relatively normal. While most of the metaphases evaluated in the passage 6 cells were in the tetraploid range (69% contained 90+ chromosomes) in the passage 17 clonal isolate BEP2D (Fig. 1D), most of the cells were near diploid (74% of metaphases with 46-52). The exact chromosome counts provided in Table 2 are primarily from the near diploid metaphases.

Cytogenetic analysis of the BEP3D cell line was performed at passage 14 (Tables 1 and 2). No normal chromosomes 7 or 11 were observed.

DNA samples extracted from the donor’s normal lung tissue and from each of the cell lines at different passages were evaluated by DNA fingerprint analysis. Samples from several other individuals were included for comparison. Analysis by hybridization to multilocus probes 33.15 (Fig. 3) or 33.6, or the combination of single-locus probes MSI, MS31, MS43, and g3 (data not shown) revealed that the donor DNA sample and samples from different passages of each cell line had the same pattern and that pattern was different from each of the control samples.

Southern Analysis for HPV18. DNA samples extracted from mass cultures of BEP1 or BEP2 cells at the fifth or fourth passage, respectively, were analyzed by Southern hybridization after either no restriction digestion, or digestion with ///Will which does not cut within HPVI8, or Pvull which cuts close to either side of the E6-E7 region (Fig. 4/4).

When the DNA fragment corresponding to the E6/E7 open reading frame was used as a probe, in the undigested samples only high molecular weight signal was observed. The apparent quantitative difference between BEP1 and BEP2 undigested was due to different amounts of total DNA loaded. In the PvulII
digest, a band at 2.2 kilobases was observed. Interestingly, when the entire pBR322-HPV18 plasmid was used as a probe, two prominent bands were observed in the HindIII-digested BEP1 cell line DNA, while at least four bands were observed in the BEP2 cell line DNA (data not shown). The bands observed following hybridization with the E6/E7 probe appeared to be one-half of pairs observed following hybridization with the full plasmid probe. This indicates that a HindIII site is present in the integrated transfected DNA, and that the DNA on either side of the HindIII site hybridizes to the full plasmid probe, but DNA on only one side hybridizes to the E6/E7 probe. It is likely that a HindIII site from the pBR322 plasmid has been included in the integrated transfected DNA. Because these data were determined on DNA from mass cultures of BEP1 and BEP2, it is possible that more than one integration is occurring in the same clone, or that integration has occurred in one location in one clone and another site in another clone.

Slot blot analysis of BEP1 and BEP2 DNA compared to DNA from the C4-1 cell line which contains a single copy of HPV18, indicates that each of these lines contains a low number of copies or a single copy of HPV18 DNA (data not shown). This is consistent with Southern data which suggest that there are one or two sites of integration in each of the cell lines.

Samples of DNA extracted from donor tissue, from a clonal isolate of the cell line, BEP3D at the 12th passage, and from the Siha cell line were electrophoresed following no enzymatic digestion, or digestion with BamHI or HindIII restriction endonucleases (Fig. 4B). BamHI linearizes the HPV16-pUC18 plasmid, whereas HindIII does not digest within this plasmid. In the undigested sample, the probe hybridized to very high molecular weight DNA. In the BamHI-digested sample, signal was detected at two prominent bands of approximately 8.2 and 3.3 kilobases in size. In addition, there were multiple fainter bands at different migration distances. In the HindIII-digested sample, the signal was detected at very high molecular weight, at approximately the same migration distance as the undigested sample. A slot blot comparing signal in BEP3D to that in Caski which contains about 50–100 copies of HPV16 and Siha (which contains 1 copy) is consistent with the presence of 10–20 copies of HPV DNA in BEP3 cells (data not shown). Taken together with the Southern data, multiple copies of HPV16 genome are present in BEP3, probably integrated into the genome as a concatemer.

Northern Analysis. Total RNA samples from mass cultures of the BEP1 (passage 9) and BEP2 (passage 7) cell lines and from HeLa (data not shown) were hybridized to a radiolabeled fragment of DNA corresponding to the HPV18 E6/E7 open reading frame (Fig. 5), and total RNA samples from BEP3D (passage 17) and from the Caski cell line previously reported to bear many copies of HPV16 (23) were evaluated by hybridi-
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The authenticity of the E6 and E6* bands was confirmed by restriction digestion, resulting in bands of the expected size. In early passage BEP1 and BEP2 cell lines, only the E6* message was clearly observed (Fig. 6A). However, at passage 13, the complete transcript and the shortened transcript were observed at approximately equivalent levels (Fig. 6C). Because of the apparent "absence" of full length E6/E7 transcript in early passage BEP1 and BEP2 cells, experiments were conducted with coamplification of known amounts of E6* and E6/E7 DNA. Amplification of the two bands closely depended on the initial ratio of cDNA. When the cDNA ratio was 1:1, amplification of the long transcript was very poor. Equivalent fmol amplification products of short and long transcripts were obtained when the cDNA short:long ratio was approximately 1:5.

Fig. 4. Southern analysis of HPV genome in HPV18 (A) or HPV16 (B) transformed bronchial epithelial cell lines. Cellular DNA was extracted from HPV16- or HPV18-transfected cultures. A, donor, BEP1 (passage 5), or BEP2 (passage 4) DNA samples undigested (UD) or digested with HindIII, PvuII, or EcoRI were hybridized to a probe homologous to the E6/E7 open reading frame. kb, kilobase. B, donor, BEP3D (passage 12), or Siha, DNA samples undigested or digested with BamHI or HindIII which do not cut within the HPV genome were hybridized to the nick-translated 32P-labeled HPV16 plasmid. Kbp, kilobase pair.

Reverse Transcriptase-Polymerase Chain Reaction Analysis of E6 Transcription. We evaluated E6 transcription by reverse transcriptase-polymerase chain reaction in early (passage 4) and late (passage 13) mass culture samples of BEP1 and BEP2 cell lines, and in passage 8 of a clonal isolate of BEP3 cells (Fig. 6). The authenticity of the E6 and E6* bands was confirmed by restriction digestion, resulting in bands of the expected size. In early passage BEP1 and BEP2 cell lines, only the E6* message was clearly observed (Fig. 6A). However, at passage 13, the complete transcript and the shortened transcript were observed at approximately equivalent levels (Fig. 6C). Because of the apparent "absence" of full length E6/E7 transcript in early passage BEP1 and BEP2 cells, experiments were conducted with coamplification of known amounts of E6* and E6/E7 DNA. Amplification of the two bands closely depended on the initial ratio of cDNA. When the cDNA ratio was 1:1, amplification of the long transcript was very poor. Equivalent fmol amplification products of short and long transcripts were obtained when the cDNA short:long ratio was approximately 1:5.

Fig. 5. Expression of HPV sequences in HPV18 (BEP1, BEP2) transfected cell line. Northern blot of human bronchial fibroblast, BEP1, or BEP2 RNA hybridized to the HPV18 E6/E7 specific probe described in Fig. 6. Kbp, kilobase pair.
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DISCUSSION

As has been described for keratinocytes (39), following transfection with HPV16 or HPV18, each of these bronchial epithelial cell lines described in this report went through two apparent stages during the immortalization process. For the HPV18-transfected lines, BEP1 and BEP2, the first stage was apparent between the third passage, when the control cells ceased dividing, and the ninth passage. During this time, a part of the population was in colonies of small rapidly dividing cells, while the rest of the cells were apparently undergoing terminal squamous differentiation. The cell population was unable to attain confluence during this stage. Following the ninth passage, the cultures consisted of a homogeneous population of small, rapidly dividing cells that were able to maintain confluence for more than three months. We hypothesize that a particular clone in each line acquired a cellular genetic alteration allowing greater resistance to terminal differentiation and took over the respective cultures.

In association with the acquired resistance to terminal differentiation, there was a change in the prevalence of different E6/E7 transforming gene transcripts. While only the E6*/E7 transcript was observed in either BEP1 or BEP2 early passage cells, both the E6*/E7 and the full-length E6/E7 message were observed in the late passage cells. There are several possible explanations for this finding that may involve loss of normal differentiation-specific gene expression. Alternative splicing occurs in a tissue- and differentiation-specific manner for some genes (40). One theory to explain this is that there is a peptide produced in certain cells that protects particular introns from the enzymes involved in RNA processing. Thus, it is possible that a peptide that protects the E6/E7 introns is normally expressed only in differentiated human bronchial epithelial cells, but that through a cellular mutation, it becomes expressed constitutively in undifferentiated dividing cells as well, allowing production of both the full-length and processed transcripts. Alternatively, there may be increased production of HPV-specific mRNA transcripts in more differentiated cells, as there is in the more differentiated superficial cells of skin tissue (41), and constitutive production of an intron protective peptide is overcome by increased general transcription. While production of the full-length transcript may not lead to a harmful effect in fully differentiated cells that are no longer capable of cell division, it may lead to abnormal growth, such as a papilloma and/or carcinoma in the regenerative cell population. In cultured cells, such a change may result in immortalization. Data from other recent studies are consistent with this hypothesis. It has been reported that transfection of human keratinocytes with vectors capable of producing E7 protein alone increases the proliferative capacity but does not lead to immortalization (16), and transfection of keratinocytes with vectors capable of producing E6+ and E7 proteins does not lead to immortalization (12, 16), whereas transfection with vectors capable of producing E6 and E7 does.

In summary, transfection of NHBE cells with either HPV16 or HPV18 acquired both resistance to confluence-induced squamous differentiation and extended population doubling potential. Resistance to squamous differentiation was associated with increased expression of E6 transcripts, and this may have mechanistic significance. The resultant cell lines have an epithelial morphology similar to that of primary NHBE cells. Further, cells from each line contain cytokeratin 18 which is characteristic of the normal epithelial lining of the upper respiratory tract (42). Because these cell lines have now attained over 100 population doublings and have been in continuous culture for over 1 year, we consider them to be immortalized. Since these cell lines have not yet given rise to tumors more than 9 months after injection into immunosuppressed athymic nude mice, they should serve as useful models to investigate the role of carcinogens and activated protooncogenes in lung carcinogenesis.

REFERENCES


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