HPV-16, Tobacco-specific $N$-Nitrosoamine, and $N$-Methyl-$N'$-nitro-$N$-nitrosoguanidine in Oral Carcinogenesis

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ABSTRACT

We previously immortalized human oral keratinocytes by transfection with recombinant human papillomavirus type 16 (HPV-16) DNA and established two cell lines. These transfected cells were morphologically different from the normal counterpart, contained intact HPV-16 DNA in an integrated form, and expressed numerous viral genes. These cells contained lower levels of wild-type p53 protein and higher levels of c-myc mRNAs compared to normal cells. However, they proliferated only in keratinocyte growth medium containing a low level of calcium and were not tumorigenic in nude mice.

A HPV-16-immortalized cell line was exposed to either 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or $N$-methyl-$N'$-nitro-$N$-nitrosoguanidine. Four chemically transformed cell colonies were isolated. These cells proliferated well in Dulbecco's minimum essential medium containing a physiological level of calcium. They contained, similar to the immortalized counterpart, integrated HPV-16 sequences and lower levels of both wild-type p53 protein and DCC messages compared to normal cells. Among the chemically transformed cells, two colonies obtained from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone exposure demonstrated an enhanced proliferation capacity in nude mice and transcribed a substantially higher amount of HPV-16 E6/E7, epidermal growth factor receptors, and c-myc genes compared with the immortalized counterpart. These experiments indicate that malignant transformation of oral keratinocytes can be caused by a sequential combined effect of "high risk" HPV and tobacco-related carcinogens.

INTRODUCTION

HPV infection is closely associated with the development of female genital epithelial cancers. Over 90% of cervical cancer biopsies contain "high risk" HPV DNA such as types 16 (HPV-16) and 18 (HPV-18) (1, 2). Similarly, HPV infection is also closely linked to benign and malignant oral lesions (3). Recent studies show that up to 30–40% of oral cancer biopsies contain the viral DNA (4). Inasmuch as the oral mucosal epithelium resembles the female genital tract and is continuously challenged by innumerable environmental influences, close association between HPV infection and oral malignancies is not surprising.

Further evidence of the role of HPV in carcinogenesis derives from its transforming capacity. Transformation of human normal foreskin and exocervical, cervical, and oral epithelial cells, the primary in vivo target cells for HPV infection, were established with cloned HPV-16 and -18 DNA (5–8). These transformed cells are immortal and harbor integrated viral DNA expressing various HPV messages including E6/E7 mRNAs. Although chronic propagation of HPV-immortalized human skin keratinocytes can lead to a malignant phenotype (9), HPV-immortalized epithelial cells are, for the most part, nonmalignant in nude mice. Furthermore, the nonmalignogenicity of HPV-immortalized human oral keratinocytes is not altered by propagation of the cell cultures in more than 80 passages (10).

The ubiquity of HPV infections, the regression of most HPV-induced dysplasias, and the long incubation period between initial infection and development of cancer indicate that HPV infection by itself may not be sufficient for neoplastic conversion of normal oral keratinocytes (11). Various other environmental agents, including chemical carcinogens, are likely to participate in the malignant progression of HPV-infected cells in the oral cavity (10). HPV-immortalized human oral keratinocytes harboring "high risk" HPV DNA may therefore be a suitable model system for investigating the sequential combined tumorigenicity of "high risk" HPV and other environmental factors.

Many epidemiological studies indicate that smokeless tobacco is linked to an increased incidence of oral and pharyngeal cancer in humans (12–15). This linkage is further supported by the presence of tobacco-specific $N$-nitrosoamines in smokeless tobacco (16, 17). Among tobacco-specific $N$-nitrosoamines, smokeless tobacco contains a high level of $N$-nitrosornornicotine and NNK, which are strong carcinogens in animals (18). However, although chronic exposure to chemical carcinogens in tobacco users is still the major known cause of oral cancer, only a minority of tobacco users develop oral cancer. Thus, an involvement of other contributing factors, e.g., ethanol and viruses, have been proposed to explain the development of smokeless tobacco-related oral malignancies (19, 20).

In the present study, we propose a hypothesis that oral cancer is induced by sequential exposure of normal oral keratinocytes to "high risk" HPV and tobacco-related carcinogen(s). To test the hypothesis, NHOK and an HPV-16-immortalized oral keratinocyte cell line, HOK-16B (8), were exposed to either NNK or MNNG. Four chemically transformed cell colonies were cloned from the HOK-16B cells exposed to chemical carcinogens. The in vitro proliferation characteristics, in vivo tumorigenicity, and the expression of HPV-16 E6/E7, TGF-α, EGFR, c-myc, p53, and the gene deleted in colon cancer (DCC) were studied from the chemically transformed cells. Since NNK mutates p53 and c-Ki-ras2 genes resulting in cell transformation (21–23), mutations of these genes were also investigated from the chemically transformed cells. Such transformants proliferate well in a culture medium containing a physiological level of calcium (1.5 mM). At least two transformants demonstrated enhanced growth capacity in nude mice, whereas primary human oral keratinocytes exposed to chemical carcinogens failed to show evidence of transformation. Cells with enhanced in vivo growth capacity contained a significantly higher amount of HPV-16 E6/E7, EGFR, and c-myc messages compared with the immortalized counterpart. No mutations in p53 and Ki-ras genes were found from the chemically transformed cells. These data indicate that HPV-16 and tobacco-related carcinogens sequentially transform oral keratinocytes into a malignant phenotype.

MATERIALS AND METHODS

Culture of Primary NHOK and HOK-16B Cells. Excised retromolar tissue from the oral cavity of a healthy male volunteer was washed in calcium and magnesium-free Hanks' balanced salt solution (GIBCO/BRL, Grand Island, NY). Monolayer cultures of primary NHOK from the tissue were
prepared as described elsewhere (8). An HPV-16-immortalized oral keratinocyte line, HOK-16B, was cultured in keratinocyte growth medium (Clonetics, San Diego, CA) supplemented with pituitary extract as described previously (8).

**Exposure of NHOK and HOK-16B Cells to NNK or MNNG.** The primary NHOK and HOK-16B cells were plated at 2 × 10^5 cells/60-mm Petri dish. When the cultures reached 70% confluency, the cultures were exposed to chemical carcinogens (three from NNK exposure and one from MNNG treatment) and named 16NNK-1, 16NNK-2, 16NNK-3, and 16MNNG-1.

**Determination of Cell Proliferation Rate in DMEM.** Confluent cell monolayers in 100-mm Petri dishes were trypsinized and counted. The cells were suspended in DMEM supplemented with 10% fetal bovine serum and 2 × 10^5 cells were plated onto one 60-mm Petri dish. The number of cells was counted after 2, 4, and 6 days of incubation at 37°C. There were three cultures in each group at each time.

**Determination of In Vivo Tumorigenicity of Cells.** The in vivo tumorigenicity of cells was determined in nude mice. Monolayer cultures were trypsinized and resuspended in culture medium and 0.1 ml of phosphate-buffered saline containing 1 × 10^6 cells were injected s.c. into athymic nude mice (nu/nu; UCLA Nude Mice Facility, Los Angeles, CA) at 24 hours after X-irradiation (300 rads). All mice received injections in the right flank and were monitored twice weekly for the appearance of tumors over a period of 4 months. Tumors that developed were excised for histological examination.

**Northern Analysis.** To determine the transcription of HPV-16 E6/E7, EGFR, TGF-α, c-myc, p53, DCC and β-actin genes, cytoplasmic polyadenylated RNA was extracted from cells using standard procedures. Probes used for analysis included: 1.2-kbp fragment (nucleotides 24–880, 3357–3820) representing the major early HPV-16 message including E6/E7 genes, human EGFR cDNA (ATCC, Rockville, MD), human TGF-α cDNA (ATCC), c-myc proviral DNA (ATCC), p53 cDNA (from Dr. E. Harlow, MGH Cancer Center, Charlestown, MA), DCC cDNA (from Dr. B. Vogelstein, Johns Hopkins University, Baltimore, MD), and the human β-actin gene (from Dr. L. Redes, Stanford University, Palo Alto, CA). All were labeled with [32P]dCTP (ICN Radiochemicals, Irvine, CA) by multiprime labeling (Amersham Corp., Arlington Heights, IL). Specific radioactivities of labeled probes were always higher than 5 × 10^6 cpm/μg of DNA. Northern blot hybridization was carried out under stringent conditions as described previously (10). After hybridization, the filter was autoradiographed on SB-5 X-ray film (Eastman Kodak, Rochester, NY) for 12 h at −70°C. After exposure, the probe was stripped off the filter for rehybridization to the next radiolabeled probe.

**Southern Analysis.** High molecular weight cellular DNA was extracted from cells by phenol/chloroform/isooamyl alcohol (25:24:1) and ethanol precipitation. To determine the presence and physical state, if any, of HPV-16 DNA, 10 μg of DNA were digested with BamHI or EcoRV restriction en- zymes. BamHI enzyme separates vectors from HPV-16 sequences, whereas EcoRV does not digest pHPV-16 plasmid that was originally transfect ed to primary NHOK to establish the HOK-16B cell line (8). The fragmented DNA were run in a 0.8% agarose gel, transferred to a nitrocellulose filter, and hybridized to 32P-labeled 7.9-kbp HPV-16 DNA under stringent conditions. After a washing, the filter was exposed to SB-5 X-ray film.

**Western Analysis.** Cellular proteins were lysed in lysis buffer [10 mM NaHPO4 (pH 7.2), 9 mg/ml NaCl, 1% Triton X-100, 5 mg/ml deoxycholate, 1 mg/ml SDS, 2 mg/ml sodium azide, and 40 μg/ml sodium fluoride] on ice for 30 min. The cell lysate was centrifuged at 14,000 rpm for 20 min and the supernatant containing 1.0 mg/ml of protein was denatured by boiling for 2 min in a sample buffer [62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 1% sodium dodecyl sulfate, 1% β-mercaptoethanol, and 0.001% bromophenol blue]. An aliquot of the denatured supernatant containing 100 μg of protein was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto an immobilized-P membrane (Millipore Corp., Bedford, MA). After incubation in blocking buffer (0.2% 1-block, phosphate buffered saline, and 0.05% Tween 20) for 3 h at room temperature, the membrane was exposed to mouse anti-human monoclonal antibody for p53 (AB-2; Oncogene Sciences, Manhasset, NY) at room temperature for 1 h. The membrane was then treated with anti-mouse IgG alkaline phosphatase conjugate (Tropix, Inc., Bedford, MA), washed with blocking buffer and assay buffer (0.1 mM diethanolamine and 1.0 mM MgCl2), incubated in Nitroblue Reagent, washed again with assay buffer, and incubated in chemiluminescent substrate solution using the Western-Light kit (Tropix, Inc.). The membrane was autoradiographed on SB-5 X-ray film for 4 min at room temperature.

**Reverse Transcription-PCR Analysis, Cloning, and DNA Sequencing.** The synthesis and amplification of p53, c-Ki-ras2, DCC, and cellular β-actin cDNAs were carried out using a RNA PCR kit (Perkin-Elmer Cetus, Irvine, CA). In a total volume of 20 μl, 10 ng of polyadenylated RNA were dissolved in a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 0.01% gelatin, 1 mM concentrations each of the four deoxyribonucleotide triphosphates, 2.5 μM oligodeoxynucleotidyldate primer, 1 unit of RNase inhibitor, and 2.5 units of reverse transcriptase. Reverse transcription mixture was incubated at 42°C for 15 min, at 99°C for 5 min, and at 5°C for 5 min. For p53 and c-Ki-ras2, the resulting cDNA product was amplified by the addition of 80 μl of PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl2, 2.5 units of recombinant Taq DNA polymerase, and 0.5 μM p53 or c-Ki-ras2 primers. The p53 sense primer extends from nt 296 to nt 315 and the antisense primer extends from nt 1015 to nt 996 of the cDNA (sense primer: 5′-CCCGAGAAAACTTACAGGCCC-3′; antisense primer: 5′-CGA- AGCGTCACGGCCAGG-3′). The c-Ki-ras2 sense primer extends from nt 3 to nt 22 (5′-GACTGAATATACACAAGG-3′) and the antisense primer extends from nt 351 to nt 332 (5′-CAGAAAAGAGCCCTCCCA-3′) of the cDNA. The amplified c-Ki-ras2 cDNA includes codons which are most frequently mutated in cancer cells, i.e., codons 11, 12, 13, 59, and 61. Each amplification cycle consisted of 1 min of denaturation at 94°C, followed by 2 min of annealing at 60°C and 3 min of extension at 72°C. A total of 35 cycles were run with a final extension step at 72°C for 7 min. Amplified p53 cDNA was ligated to PCR vector using the TA Cloning Kit (In Vitrogen, San Diego, CA) under the conditions recommended by the manufacturer. The nucleotide sequence of the amplified cDNA was determined by the primer extension method as described previously (10). Five p53 and four c-Ki-ras2 clones were sequenced for each cell line.

**The DCC cDNA was amplified using sense primer (5′-AACAGAGGATTCAACCAAT-3′) from exon 22 and antisense primer (5′-AGAGCAGTAAACTTGGACAG-3′) from exon 23 (24). The cellular β-actin cDNA was amplified using sense primer (5′-ATCATGTTTGAGACCTACA-3′) and antisense primer (5′-CATCCTCGTGGATGCTC-3′) (25). Each amplification cycle consisted of 1 min of denaturation at 94°C, followed by 2 min of annealing at 56°C and 3 min of extension at 72°C. A total of 35 cycles were run with a final extension step at 72°C for 7 min. The amplified cDNA was electrophoresed in 1.5% agarose gel, transferred to a nylon filter, and hybridized to internal oligonucleotide probes (DCC cDNA, 5′-GAGATGATGTGGCAACA-3′ and β-actin cDNA, 5′-GACCTGGTGGCGCCGGCAGTTGCT-3′) after 3′-end labeling with [32P]dATP using T4 polynucleotide kinase (GIBCO/BRL). The hybridization was carried out as described in “Southern Analysis.” The oligonucleotide primers and probes obtained from Bio-Synthesis, Inc. (Denton, TX) were purified and desalted before use.

**RESULTS**

**Morphology and Proliferation Rate.** The chemically transformed cells were morphologically similar to their immortalized counterpart and proliferated well in DMEM supplemented with 10% fetal bovine serum. Population doubling times of the 16NNK-1, 16NNK-2, 16NNK-3, and 16MNNG-1 cells in DMEM were approximately 32, 38, 48, and 42 h, respectively, but the HOK-16B line did not proliferate in this medium (Fig. 1). The HOK-16B cells eventually differentiated and detached from the culture dishes. Primary NHOK were able to be subcultured for up to the 4th passage in keratinocyte growth medium but the cells began to differentiate and completely detached from the culture plates by passage 5. Further, NHOK could not be transformed by NNK or MNNG exposure. This experiment was repeated five times and the same results were consistently obtained.

**In Vivo Tumorigenicity.** No tumors developed in mice receiving NHOK, HOK-16B, 16NNK-1, or 16MNNG-1 lines. However, 60 and

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Fig. 1. Chemically transformed HOK-16B cells have obtained the ability to grow in DMEM supplemented with 10% fetal bovine serum.

80% of animals receiving the 16NNK-2 and 16NNK-3 cell lines developed tumors, respectively. Tumors began to appear at 3 weeks after injection and reached their peak at 0.5 cm in diameter by 7 weeks. The tumors then regressed, leaving only necrotic tumor tissue by 12 weeks. Microscopically, the tumors exhibited a differentiated squamous cell carcinoma histology.

**Viral DNA and RNA.** Southern analysis after BamHI digestion showed that the immortalized and chemically transformed cells contained 7.9-kbp HPV-16 DNA genome, indicating the presence of intact HPV-16 DNA in these cells. In addition to the 7.9-kbp HPV DNA genome, all of the cells contained rearranged HPV-16 DNA sequences; the hybridization of cellular DNA digested with BamHI showed HPV-16-specific bands larger than 7.9 kbp (Fig. 2A). After digestion with EcoRV, an enzyme that does not cut the p11HPV-16 plasmid (8), the Southern analysis showed HPV-16 specific bands larger than 30 kbp, suggesting that HPV DNA exists as an integrated form, not as an episomal form, in both the parental (HOK-16B) and all chemically transformed oral keratinocytes (Fig. 2B).

Northern analysis using the probe containing HPV-16 E6/E7 gene showed that HOK-16B cells expressed the 1.6-kilobase HPV-16 E6/E7 mRNAs. Chemically transformed cells also expressed numerous mRNAs including the predominant 1.6-kilobase E6/E7 messages. The densitometric intensity of the 1.6-kilobase E6/E7 bands of the tumorigenic 16NNK-2 and 16NNK-3 cell lines was notably greater than that of the parental cell line but when compared to the HOK-16B cell line, the intensity of these bands of the nontumorigenic 16MNNG-1 and 16NNK-1 cells was similar for 16MNNG-1 but significantly lower for 16NNK-1 (Fig. 2C; Table 1).

**Analysis of p53 and c-Ki-ras2.** The levels of 2.7-kilobase p53 mRNA transcript in the immortalized and chemically transformed cells were similar to each other (Fig. 3C; Table 1). No mutations were found in the region of p53 and c-Ki-ras2 cDNA amplified from NHOK or any of the established cell lines. Western blot analysis showed that p53 protein levels in the immortalized and chemically transformed cells were similar but notably lower than that from NHOK (Fig. 4).

**Expression of TGF-α, c-myc, EGFR, and the Gene DCC.** The 4.5-kilobase mRNA is the common transcript of TGF-α gene in normal, immortalized, and chemically transformed oral keratinocytes. The 1.5-kilobase TGF-α mRNAs were also seen in the immortalized and chemically transformed cells. Among these transcripts, the amount of 1.5-kilobase TGF-α mRNAs was notably enhanced in 16NNK-2, 16NNK-3, and 16MNNG-1 cells (Fig. 3A; Table 1). Two c-myc transcripts with sizes of 2.4 and 1.1 kilobases were expressed from both the HOK-16B and the chemically transformed cells, but the amount of this gene transcription was substantially higher in the chemically transformed cells than in the HOK-16B line (Fig. 3B; Table 1). The expression of both 10.0- and 5.0-kilobase EGFR messages from the chemical transformants was also significantly increased compared to the parental counterpart (Fig. 3D and Table 1). The DCC mRNAs were not determined from any type of cells when analyzed by Northern analysis (data not shown), but they were detected by reverse transcriptase-PCR analysis from all cell types. The densitometric in-
Table 1 Relative degree of transcription of HPV-16 E6/E7, EGFR, TGF-α, c-myc, p53, and DCC genes from the HPV-immortalized and chemically transformed oral keratinocytes

<table>
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<th>Cell lines</th>
<th>HPV E6/E7</th>
<th>EGFR</th>
<th>TGF-α</th>
<th>c-myc</th>
<th>p53</th>
<th>DCC</th>
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<td>NHOK</td>
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<tr>
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<td>1.0</td>
<td>1.0</td>
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<td>4.1</td>
<td>0.7</td>
<td>0.7</td>
<td>2.0</td>
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<tr>
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<tr>
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<td>2.0</td>
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</table>

^a Numbers in parentheses, size of the messages in kilobases.
^b ND, not done.

DISCUSSION

The hypothesis that oral cancer is induced by multiple factors, including HPV infection and chemical carcinogens, is supported by the following observations. (a) Certain types of HPV are consistently associated with squamous cell carcinoma of the human oral cavity (3, 26). Although some of these viruses can occasionally be detected in histologically normal tissues, only a small fraction of HPV-infected lesions progress to cancer (27, 28). (b) Even though chronic exposure to chemical carcinogens in tobacco is still the major known cause of oral cancer, only a minority of tobacco users develop oral cancer. These observations suggest that these risk factors are involved in the development of oral cancer and/or the progression of this disease. Thus we hypothesize that oral cancer is developed by sequential exposure of normal oral epithelial cells to “high risk” HPV and tobacco-related chemical carcinogens.

To test this hypothesis, HPV-16-immortalized oral keratinocytes (8) were exposed to NNK, one of the most potent alkylating carcinogens found in smokeless tobacco, or MNNG, an alkylating agent that does not require metabolic activation (29). Inasmuch as (a) both NNK and MNNG are genotoxic agents and can alkylate DNA, often resulting in G to A transitions in rodents, and (b) NNK can pyridyloxobutylate DNA and can induce p53 and c-Ki-ras2 mutations in laboratory animals (21-23), exposure of the immortalized oral keratinocytes to these carcinogens could generate more transformed cells.

Our results show that sequential in vitro exposure of normal human oral keratinocytes to HPV-16 and NNK can generate cells with limited tumorigenicity. Although the tumors had spontaneously regressed, these cells were different from their immortal counterparts. The precise reason of tumor regression is not clear but may be due to the continued expression of wild-type c-Ki-ras2 in the chemically treated cells. However, the chemically transformed cells proliferated faster and were calcium resistant, whereas the immortalized cells terminally differentiated and died in culture medium containing a physiological level of calcium. These results demonstrate that the immortalized cells are further transformed by exposure to chemical carcinogens. The molecular mechanisms of cell transformation by these chemical car-
Although the reasons for enhanced HPV-16 E6/E7 messages in the tumorigenic cells remain speculative, mutation of the E2 gene may play an important role in the overexpression of viral E6/E7 (37). Mutations of the E2 gene at its DNA-binding domain induce the loss of the repressor activity of E2 that may result in an overexpression of E6/E7 messages. Recent studies have also implicated mutations of the viral E1 gene as having a possible role in carcinogenesis progression (28). Since HPV-immortalized and chemically transformed cells were originally transfected with psHPV-16d, a plasmid containing linear HPV-16 DNA with an interruption of the E1 gene, it is unlikely that these cells express intact E1 messages.

Detection of lower amounts of DCC messages in both the HPV-immortalized and the chemically transformed cells is also interesting. Since DCC mRNA is frequently absent or reduced in most colorectal carcinoma cell lines (38), low expression of DCC gene in these cells may be closely linked to different phenotypic characteristics of these cell lines from NHOK.

REFERENCES


Fig. 5. Southern blot hybridization analysis of amplified DCC cDNA fragment derived from NHOK, HOK-16B, and chemically transformed cell lines 16NNK-1, 16NNK-2, 16NNK-3, and 16MNN-1. The cDNAs were constructed from mRNAs using reverse transcription and the DCC fragment was amplified with polymerase chain reaction. The amplified cDNA fragments were then electrophoresed, transferred to a nylon filter, and hybridized to [32P]DCC oligonucleotide. Bp, base pair.


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