Chromosomal abnormalities in HPV-16-immortalized oral epithelial cells

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Abstract

Human papilloma virus (HPV) type 16 has an established association with anogenital carcinoma, and to some extent with human oral squamous cell carcinoma. We hypothesize that HPV type 16 is capable of inducing chromosomal and cell cycle changes in cultured oral epithelial cells. Normal human oral epithelial cells were immortalized with recombinant retrovirus containing the E6/E7 open reading frames of HPV type 16. These cells have been in culture for more than 350 passages and over 4 years. Flow cytometry demonstrated an average of 42% nuclear aneuploidy in HPV 16-immortalized cells; 16% in normal controls (probably tetrasomy). Cytogenetic analysis demonstrated significant progression of chromosomal abnormalities. Cells at early passage (p10) showed trisomy 20, with no other major changes. At passage 18, trisomy 1q and monosomy 13 were seen in addition to trisomy 20. At passage 61 there were two distinct cell populations ('a' and 'b'), with multiple chromosomal changes including trisomy 5q,14,20 in one line and 7p,9q,11q in the other. Both populations had monosomy 3p, with monosomy 8p in one population and monosomy 13 in the other. At passage 136, the cells were essentially identical to population 'b' of passage 61. At this passage, mutation of the p53 gene was detected at codon 273 of exon 8, with G to T conversion (Arg to Leu). This was absent in the normal cells from which this line was developed. Passage 262 contained the two major cell populations, each with a sub-group with additional chromosomal changes such as 10p monosomy. Cells from passages 217 and 305 were injected into nude mice a year apart. Both failed to produce tumors, as did normal cells. In conclusion, we present an HPV type 16-immortalized oral epithelial cell line (IHGK) with extensive and progressive chromosomal abnormalities, invasive growth in culture and yet no tumor formation in nude mice. We suggest that the question as to whether HPV alone can induce transformation is still open.

Introduction

It is generally accepted that carcinogenesis is a multi-hit process, involving a number of aberrant genetic changes and culminating in malignant transformation (1). There can be abnormal products or over-production of growth factors, recep-

tors or genes controlling signaling from cell surface to nucleus (2). Oncogenes are probably involved in both initiation and progression of neoplasia (3).

The role of human papilloma virus (HPV*) in the pathogenesis of anogenital and oral cancer has been studied extensively at the clinical, epidemiological and experimental levels. HPV is a site-specific DNA virus that is known to infect the basal cell layer and replicate during epithelial cell differentiation. Of the more than 70 types of HPV that have been identified, 22 are related to malignant lesions that include cervical and oral cancers. Between 85 and 90% of cervical cancers and high-grade cervical intraepithelial neoplasia contain HPV DNA, with types 16 and 18 most common (4-7). Similarly, up to 90% of oral cancers have been reported to contain HPV DNA, again with types 16 and 18 predominating (8). E6 and E7 oncoproteins are consistently expressed in HPV-positive cervical carcinomas and cell lines (9-11). Their gene products are transforming proteins that can form complexes with both the retinoblastoma and p53 tumor-suppressor gene products (12,13).

HPV 'high risk' types are 16 and 18, while low risk types are 6b and 11. Types 16, 18 and 33 are most prevalent in malignant cells (14). Type 18 is generally associated with glandular tumors and type 16 with squamous tumors (15). Type 18 is associated with more aggressive cervical tumors than type 16 (15,16) and with younger age at diagnosis and greater frequency of lymph node metastasis (17).

Immortalization of cells following transfection with HPV 16 DNA is a reproducible phenomenon that occurs with a high frequency independent of the genetic characteristics of the host cells (18,19). While HPV DNA may persist in episome form in benign lesions, most tumors and tumor cell lines show single or multiple integrated copies of HPV 16 or 18 DNA (20-25). Only cell lines having HPV sequences integrated into cellular DNA become permanent lines, showing that genetic alterations caused by viral DNA integration are necessary for continuous growth (19). Immortalization of human cells by viral DNA is usually associated with aneuploidy and rearrangement of chromosomes (19,26-32).

The limited knowledge of the molecular and genetic events in human oral cancer, and its relationship to HPV, is partly due to the lack of a well-established suitable in vitro model (33). If the genetic and/or molecular markers of oral cancer are known, they can be detected by non-invasive sampling of epithelium such as oral scrapings. We present an in vitro model in which normal human oral epithelial cells have been immortalized with HPV type 16 DNA E6/E7 genes (33). Cell kinetics and chromosomal changes in the cell lines are presented. For brevity, we will refer to this cell line as immortalized human gingival keratinocytes (IHGK).

Materials and methods

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) except where noted. Vitrogen (bovine dermal collagen) was purchased from Celtrix...
Monolayer culture

We followed the procedure described by Oda and Watson (34). Specimens obtained from healthy patients undergoing surgery for impacted third molar removal were washed immediately with phosphate-buffered saline (PBS). After removing excess and damaged epithelium and connective tissue, the specimens were cut into small pieces and incubated overnight in Dispase II (Boehringer Mannheim, Mannheim, Germany) at a concentration of 4 mg/ml in PBS, with agitation, at 4°C. Surface epithelium was mechanically separated and trypsinized to dissociate the cells into a single cell suspension. The cells were centrifuged and resuspended in keratinocyte basal medium (KBM) with BEGM singlequats supplements (Clonetics, San Diego, CA). The parent population used for immortalization was derived from one healthy donor.

HPV 16 Immortalization

Normal epithelial cells at 50% confluence were transfected with HPV 16 E6/E7 open reading frames (ORFs) using a recombinant retroviral system, as previously described (35). Briefly, HPV E6/E7 ORFs were cloned into the murine-based retroviral vector LXSN. The constructs were transfected into a packaging cell line PA317 and recombinant retrovirus collected in the supernatant. The resulting vector, PLXSN, was used to infect the early passage population used for immortalization was derived from one healthy donor.

DNA Extraction

DNA from human placenta was used as a positive control, as it has been confirmed to have mutation of exon 5 (39). DNA from human placenta was used as a control. DNA from human placenta was used as a control.

Fig. 1. Phase contrast microscopy of human oral epithelial cells in monolayer culture. (A) Normal cells. (B) HPV 16-immortalized cells.

Cyto genetic analysis

A single cell suspension was prepared from the monolayer cultures at the indicated passages, and cells were sub cultured in K-SFM and placed onto clean microscope slides. Following 48 h subculture, colcemid (0.1 μg/ml) was added to the culture medium for 2 h prior to chromosome harvest. The latter was prepared in a standard fashion using KCl 0.075 M as a hypotonic medium and methanol/acetic acid (3:1) as fixative. After staining by Giemsa, 20–21 cells were completely analyzed for each cell line. Two to 20 cut karyotypes were established for each cell line. Karyotypes were described using the ISCN (International System for Human Genetics Nomenclature, 1985, 1991).

Digestion with protease K and phenol/chloroform extraction were used to prepare specimens for DNA analysis and for PCR amplification (37).

Detection of p53 mutation by SSCP-PCR and sequencing: PCR primers for p53 exons 5 to 8 were used in this study, as these exons were previously shown to have a high incidence of mutations (38).

PCR amplification was performed by the standard method with necessary modifications. Briefly, the PCR reaction mixture consisted of 25 μl reaction volume containing 0.25 μg genomic DNA, 10 mM Tris–HCl, pH 8.4, 2.5 mM MgCl2, 50 mM KCl, 0.01% gelatin, 100 mM dNTPs (10 mM dCTP), 75 ng of each primer, 5 μCi of α-32P-dCTP (3000 Ci/mmol) and 1 U of Taq polymerase. After a brief spin, the reaction mixtures were heated in an automated thermal cycler (Perkin Elmer Corporation, Norwalk, Connecticut) for 35 cycles of amplification.

The PCR product was diluted 10-fold with SSC running dye containing 20 mM EDTA (pH 8.0), 0.1% xylene cyanol, 95% deionized formamide and 0.1% bromophenol blue. The reaction was heated at 95°C for 5 min., immediately chilled on ice, and loaded onto a non-denaturing gel consisting of 8% acrylamide, 5% (v/v) glycerol and 0.5% TBE buffer. Samples were electrophoresed at room temperature in 0.5% TBE running buffer. After migration, the gels were dried on filter paper and exposed to Kodak XAR X-ray film at room temperature overnight without an intensifying screen. The C33A cell line DNA was used as a positive control, as it has been confirmed to have mutation of exon 8 (39). DNA from human placenta was used as a negative control for each analysis. Direct sequencing of single-stranded PCR products was carried out by Taq dyeodeoxy Terminator Auto Cycle Sequencing System (Applied Biosystems). At least two independent PCR products were sequenced in each case.

Tumorigenicity

At passages 217 and 305, 107 HPV-immortalized human oral cells (in a total volume of 0.1 ml) were injected subcutaneously once into the backs of two
and 42% near-tetraploid (Figure 2B), compared to the normal control showed focal positive staining along the basal cell layer as expected in normal epithelial cells after birth (33). The normal control cells maintained the usual pattern of cell maturation.

When stained with keratin 19, an embryonal keratin found in premalignant and malignant epithelial cells (57), the IHGK cells stained strongly and uniformly positive while the normal premalignant and malignant epithelial cells (57), the IHGK cells were pleomorphic with high mitotic activity and more differentiated cells (B). By organotypic culture, the IHGK cells have been in culture for over 4 years and more than 350 passages, while the normal control survive between seven and nine passages (33). By phase contrast microscopy (Figure 1) they are small, uniform and basaloid with predominant basaloid cells interspersed with larger, more differentiated cells (B). By organotypic culture, the IHGK cells are 58% near-diploid and 42% near-tetraploid (Figure 2B), compared to the normal control cells, which are 84% diploid and 16% tetraploid (Figure 2A). The karyotype of the normal cells was consistently normal, and the 16% aneuploidy is not unusual in cultured cells, and is probably due to tetraploidy.

On cytogenetic analysis, the IHGK cells showed numerous chromosomal abnormalities, summarized in Tables I, and in Figures 3A and B. All 20 cells karyotyped at each passage showed similar abnormalities. Therefore, the karyotypes indicated are composite to reflect the common abnormalities. Most passages showed trisomy 20, however it was absent from population ‘b’ at passage 61, passage 136 and the ‘b’ population at passage 262. Monosomy 3p and 13 were noted in the middle passages (the latter indicating possible loss of Rb tumor suppressor gene) together with multiple chromosome rearrangements. The number of chromosomal abnormalities increased throughout, unlike the HPV-immortalized cell lines reported by Smith et al. (40). Cells at passage 61 clearly showed two chromosomally different cell populations, which persisted up to passage 262, although at passage 136 only one population (b) was observed. At passage 262, subgroups of populations ‘a’ and ‘b’ had developed with characteristic additional chromosomal changes such as monosomy 10p. The control normal cells, derived from the oral epithelium of a male and not infected with HPV, had a normal 46, XY karyotype.

**p53 mutations**

With the SSCP–PCR technique, passage 136 cell line was screened for point mutations in exons 5–8 of the p53 gene.
Table I. Karyotypes and common chromosomal changes of HPV 16-immortalized oral epithelial cells at different passages with progression in culture

<table>
<thead>
<tr>
<th>Cell Passage</th>
<th>Trisomy</th>
<th>Monosomy</th>
<th>Rearrangements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Passage 10: 45-49, XY, add (13)(p11), +20(cp20)</td>
<td></td>
<td></td>
<td>13p11</td>
</tr>
<tr>
<td>Passage 18: 44-48, XY, i(8q), +9, der(11)(11:11)(q21;q23), -13, add (14)(p11), +16, add(19)(p13.3), +20, +22(cp20)</td>
<td>1q, 20</td>
<td>13</td>
<td>1q21,11q23</td>
</tr>
<tr>
<td>Passage 61:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population a: 45-47, XY, der(14)(3;14)(p11:p11), +der(3)(3;14)(q10;q10), der(8)(8;21)(p11;q11), 14, +20,der(22)(5;22)(q13;q13)(cp16)</td>
<td>3q, 14, 20</td>
<td>3p, 8p</td>
<td>3p11, 3q10, 5q13, 8p11, 14p11, 14q10, 2q11, 22q13</td>
</tr>
<tr>
<td>Population b: 43-44, XY, der(3)(3;7)(q10;q10), der(16)(9;16)(q11;q24), der(20)(9;20)(q11;q13), add(9)(q12), -13, der(14)(14;15)(q10;q10), -15, der(22)(11;22)(q11;13)(cp5)</td>
<td>7p, 9q, 11q</td>
<td>3p, 13</td>
<td>2q10, 7q10, 9q11, 11q11, 14q10, 5q10, 16q24, 20q13, 22q13</td>
</tr>
<tr>
<td>Passage 136: 45, XY, der(3)(3;7)(q10;q10), add(9)(q12), -13, add(14)(p11), der(16)(9;16)(q11;q24), der(20)(9;20)(q11;q13), der(22)(11;22)(q11;13)(cp10)</td>
<td>7p, 9q, 11q</td>
<td>3p, 13</td>
<td>2q10, 7q10, 9q11, 11q11, 14p11, 16q24, 20q13, 22q13</td>
</tr>
<tr>
<td>Passage 262:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population a: 42-45, XY, -Y, -3 or add (3) (p22), add (5) (q11), der (8) t (8;21) (p11;q11), add (9) (q12) or del (9) (7p23), i (10q), -10 or del (10) (q22), der (11) t (9;11) (q22;p15), der (14) t (3;14) (p11:p11), add (19) (q13), der (20) t (9;20) (q11;q13), +add (20) (q13), -21, der (22) t (5;22) (q13;q13)(cp11)</td>
<td>3q, 20</td>
<td>3p, 8p, 10p, -Y</td>
<td>3p22, 3p11, 5q11, 5q13, 8p11, 9q12, 9q11, 9q22, 10p11, 11p15, 14p11, 19q13, 20q11, 20q13, 21q11, 22q13</td>
</tr>
<tr>
<td>Sub-population a-1: 42-45, idem, del (1) (q11), +i(1q), -del(9), -add(20)(cp3)</td>
<td>1q, 9p</td>
<td></td>
<td>3p22, 3p11, 5q11, 5q13, 8p11, 9q12, 9q11, 9q22, 10p11, 11p15, 14p11, 19q13, 20q11, 20q13, 21q11, 22q13</td>
</tr>
<tr>
<td>Population b: 43-46, XY, add(3)(p12), add(9)(q12), -11, add(13)(p11), add(14)(p11), der(16)(9;16)(q12;q24), der(20)(9;20)(q11;q13), der(22)(11;22)(q11; q13)(cp)</td>
<td>9q</td>
<td>3p, 11p</td>
<td>3p12, 9q11, 9q12, 11q11, 13p11, 14p11, 16q24, 20q13, 22q13</td>
</tr>
<tr>
<td>Sub-population b-1: 42-45, idem,-add(3), +der(3)(3;7)(q10;p10), der(19)(7;19)(q11;q1) (cp2)</td>
<td></td>
<td></td>
<td>3p12, 9q11, 9q16, 11q11, 13p11, 14p11, 16q24, 20q13, 22q13</td>
</tr>
</tbody>
</table>

The wild type and mutation shifts of p53 are illustrated in Figure 4. A mutation band was seen as an extra band together with the wild type bands. The reason for the presence of wild-type bands may be the presence of a small number of normal cells, or heterogeneity in the tumor population. Mutation of p53 at exon 8 was detected in the cell line. No such mutation was present in the normal cells from which the immortalized line was developed.

The mutation detected from the passage 136 cell line was confirmed to be a point mutation at codon 273 of exon 8 representing a G to T transversion (Arg to Leu).

Tumorigenicity

The cultured cells were tested for tumorigenicity by subcutaneous injection of $10^7$ cells per animal into nude mice, using both immortalized and normal cells. This was carried out at passage 217 and again at passage 305 of the IHGK cells. In neither case did either cell type result in development of tumor. The mice from the first study were still alive and healthy more than 6 months later.

Discussion

Our results demonstrate that HPV 16 E6/E7 gene immortalizes oral epithelial cells and leads to progressive chromosomal changes, but apparently does not result in tumorigenicity, as assessed by the nude mouse technique.

HPV infection alone does not necessarily lead to malignancy. In a similar study, five HPV immortalized cell lines with numerous deviant and altered chromosomes were non-tumorigenic in mice (31). All had cells with either homogeneous staining regions or double minute chromosomes, alterations associated with malignancy or drug resistance. Viral sequences were found on the abnormal chromosomes at junctions of chromosome translocations, at achromatic lesions and within homogeneously staining regions and duplicated chromosome segments (31). Cytogenetic analysis of eight HPV-immortalized human foreskin keratinocyte cell lines showed all were abnormal, containing a variety of numerical and structural aberrations (40). The viral DNA was integrated and all lines had extended lifespans, but were not tumorigenic. These cell lines were clonally and chromosomally stable over extended passages (40), in contrast to our cell line, which shows progressively accumulating chromosomal defects.
While HPV infection frequently immortalizes the host cells, it usually is not sufficient on its own to transform them to a malignant phenotype. Cells stated to be transformed by HPV have usually also been irradiated with UV light (41). However, prolonged passage in culture, or co-operation with activated ras oncogene, have been shown sufficient for full conversion to a malignant phenotype (26,42–45). R30 gingival fibroblasts from a patient taking phenytoin, which had a stable translocation between chromosomes 8 and 18 and expressed a higher steady state level of c-myc, were readily transformed with HPV-16, whereas normal gingival cells were not (46). Similarly, chemical carcinogens cause neoplastic conversion of HPV-immortalized oral cells, but not normal oral cells in vitro. When cells were treated with nitromethyleurea (NMU) and TPA, only HPV-18 immortalized cells converted to a malignant phenotype, not normal cells (47). This may be due to the normal cells' ability to repair damaged DNA, an ability which is lost in the immortalized cells. Transient G1 arrest may be associated with enhanced levels of intranuclear wild type p53 protein (41). High risk HPV E6 protein binds to wild type p53 and increases degradation of p53 protein (13,48). Thus, unlike normal cells, immortalized cells readily convert to neoplastic cells because of their inability to arrest the cell cycle and repair DNA when challenged with genotoxic agents such as chemical carcinogens (41).

p53 mutations also are common in human cancer cells (49). Mutation of the p53 gene is found in human primary carcinomas of the cervix and cervical intraepithelial neoplasia containing HPV 33 infection (50). While our cells show no loss of chromosome 17p (the location of the p53 gene), nor its involvement in any of the chromosome rearrangements, we have demonstrated p53 gene mutation. This finding suggests that HPV 16 E6/E7 genes may be capable of inactivating the p53 gene not only by degrading p53 gene product, but also by causing mutation of the gene.

Among the numerous and progressive chromosomal changes monosomy 10p was clearly evident towards the late passages, i.e. passage 262. Monosomy 10p has been also reported by others with foreskin cells immortalized by HPV-18 and other human keratinocytes immortalized by HPV-16 (58,59). Pei et al. (58) suggested that chromosome 10p may be the site for a potential tumor suppressor gene. Many other chromosomal changes found in this cell line have been identified in one or more malignant human neoplasms or cultured immortalized cells (51). For example, trisomy 20 has been described in leukemia and epithelial bladder cancer, deletion of chromosome 8 is common in prostate cancer, deletion of chromosome 13 is common in retinoblastoma, and 9p monosomy indicates loss of the pl6 tumor suppresser gene. This suggests that the mechanism of immortalization in these cells has much in common with the process of malignant transformation at other sites, in that multiple genetic changes, many at the same sites, are involved. By passage 290, these cells were growing at a much more increased rate and exhibiting morphology and chromosomal changes suggestive of transformation. However, they were, at this stage, still not tumorigenic in the nude mouse assay.

While studies of genetic and molecular markers in HPV-related cervical cancer have been widely reported, such studies in oral cancer are still at an early investigational phase. A recent study showed the presence of HPV types 16 and 18 in oral epithelial biopsies (52). HPV types 6 and 11 were found to be benign in the head and neck, as in the genital region. Similarly, types 16 and 18 were found in malignant lesions, as in the genital region. Some HPVs, particularly type 16 variants, may be associated with ubiquitous asymptomatic oral infections (53) analogous to findings of HPV in uterine cervix of histologically normal women (54). Tobacco and alcohol are the main risk factors for head and neck cancers (8). Of 30 oral carcinomas studied with PCR, 27 were positive for oncocgenic HPV type 16 or 18 DNA. Almost all had a history of tobacco use (8). HPV has only recently been identified as a risk factor for oral cancer, and its role is not yet sufficiently defined to classify it as a major factor, although it is present in up to 90% of head and neck cancers (8). Nor is it yet clear how HPV interacts with tobacco and alcohol in the development of malignancy; further data are required.

Conclusions

Retroviral infection with HPV 16 E6E7 genes successfully immortalized oral epithelial cells, which have now been in culture for almost 4 years and more than 350 passages. In contrast, normal cells can be maintained in culture for only five to seven passages. In morphology, the immortalized cells are more uniform and basoloid than the normal heterogeneous morphology and routinely show invasion of the matrix.

Flow cytometry shows that the HLGK cells have a significant increase in aneuploid population, and the chromosomal changes noted in these immortalized cells are numerous and demonstrate a progression from early to late passages, characteristics of transformed cell lines. Additionally, mutation of the p53 gene was detected. While these cells fail the classic test for malignancy, tumorigenicity in nude mice, they have many of the characteristics of transformed cells. It is possible that the nude mouse test is not adequate for testing malignancy in some types of cells, as shown by Chang et al. (55) for oral squamous cell carcinoma.

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References


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