Progression of Head and Neck Cancer in an In Vitro Model

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Objective: To identify alterations in angiogenesis and cell cycle regulation as preneoplastic cells progress to cancer in an in vitro model of head and neck tumor progression.

Methods: Immortal human gingival keratinocyte (IHGK) cells (preneoplastic) were derived from normal oral keratinocytes and were immortalized with human papillomavirus 16. Transformation of IHGK cells with a carcinogen (NNK, 4-[methylnitrosamino]-1-[3-pyridyl]-1-butane) gave rise to IHGKN cells. We determined the growth rates, cell cycle phase, expression of cell cycle regulators, and expression of vascular endothelial growth factor along with the organotypic features of these cells and compared them with characteristics of head and neck cancer cells.

Results: IHGK and IHGKN cells grown in raft culture were morphologically similar to severe dysplasia and carcinoma, respectively. The proportion of cells in G0/G1 was similar between IHGK and IHGKN. However, the proportion of IHGK cells was 35% greater in S phase as compared with the IHGKN cells, while a greater percentage (40%) of IHGKN cells were in G2/M. The expression of the other cell cycle regulators tested was unchanged. IHGK cells secreted less vascular endothelial growth factor on day 1 when compared with IHGKN (50.6 vs 245.6 pg/mL), along with a lower overall production rate (79% vs 133%).

Conclusions: Transformation of IHGK cells resulted in the activation of vascular endothelial growth factor associated with angiogenesis. Inactivation of the G1 cell cycle regulation occurred during immortalization and before transformation, and was sustained after carcinogen exposure. These alterations correspond to changes observed in patients with head and neck squamous cell carcinoma. This model can be useful in testing novel therapeutic and preventive strategies.

HEAD AND NECK cancer develops from the accumulation of genetic alterations through a series of progressive steps in which normal cells are transformed into preneoplastic cells and then to cancer. During this process, cancerous cells gain a growth advantage over the normal cells. The recent understanding of the molecular biology of this progression has led to the development of novel therapeutic agents and approaches. Understanding how cancer has altered regulatory pathways, such as cell cycle regulation and angiogenesis, can lead to testing of novel agents.

The cell cycle is divided into 4 phases: G1, prepare to synthesize DNA; S, synthesize DNA; G2, prepare for cell division; and M, mitotic phase. The G1 phase is a crucial checkpoint in cell cycle regulation that is altered in head and neck squamous cell carcinoma (Figure 1). Passage from the G1 phase to the S phase is regulated by cyclin-dependent kinases (CDKs). G1 is controlled by 2 pathways (Rb-CDK4 or p53-CDK2). The CDK4 is activated by cyclin D1 and inhibited by p16. The CDK2 activity is promoted by cyclin E and inhibited by p21. The p53 protein up-regulates p21 expression. The p16 inhibits MDM2. Since MDM2 increases degradation of p53, p16 inhibits MDM2. The formation of new blood vessels (angiogenesis) allows tumors to grow at
MATERIALS AND METHODS

CELL LINES AND TRANSFORMATION

The IHGK cells are normal oral keratinocytes that have been immortalized with HPV16.10 IHGK cells proliferate only in enriched keratinocyte growth medium (Defined Keratinocyte–Serum Free Media [DK-SFM]; Gibco-BRL, Grand Island, NY) with low calcium and without serum. IHGK cells with less than 100 passages were used in this study. IHGK cells were transformed with a carcinogen, NNK, by a 5-week exposure to a medium containing 36 µg of NNK per milliliter. The transformed cells were then selected with Dulbecco minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) medium, since HNSCC cell lines, but not IHGK cells, grow in this medium. The selected cell line was designated IHGKN. IHGK cells were examined at passages less than 100, since spontaneous p53 mutations are observed at passages later than 130.10 The HNSCC cell lines, HN12 and HN30, were grown in DMEM with 10% FCS. The p53 gene is mutated in HN12, whereas HN30 has a wild-type p53 gene.11 Proliferation rates were determined by means of tritium uptake in triplicate. All cells were analyzed at 70% to 80% confluence.

RAFT ORGANOTYPIC CULTURE

As previously described,13 a raft culture was performed to obtain a 3-dimensional organoid model. Sublethal irradiated NIH 3T3 fibroblasts were washed and suspended in DMEM with 10% FCS. The concentration of VEGF was determined by means of an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minn). Cells (300000) were placed in 6-well plates for each experiment in a final volume of 3 mL of culture medium. During logarithmic growth, cells were transferred to a Biocoat Deep well plate (Collaborative Biomedical Products, Bedford, Mass). Two milliliters of the mixture was added to each Biocoat Cell Culture Control Insert (Collaborative Biomedical Products, 3.0 µm) and allowed to gel at 37°C for at least 1 hour before use. Approximately 2 × 10⁵ cells (IHGK, IHGKN, HN30, and HN12) in 0.1 mL of medium were seeded on a collagen plug and incubated in a humidified atmosphere of 5% carbon dioxide at 37°C for 1 hour to allow for attachment. Once the majority of the cells were attached, the insert and well were flooded with cell culture medium (DK-SFM) and incubated in a humidified atmosphere of 5% carbon dioxide at 37°C until confluence was reached, usually overnight.

Cell culture medium covering the cells in the insert was then carefully removed, and the insert was transferred to a Biocoat Deep well plate (Collaborative Biomedical Products) to allow the cells to grow at the air-liquid interface. To induce stratification and differentiation of these cells, 10 mL of differentiation medium consisting of a 1:1 mixture of DK-SFM and enriched DMEM/F-12 (3:1 mixture of DMEM and Hams F-12 with 10% FCS and penicillin, streptomycin, and fungizone [PSF]; Gibco-BRL) was added to the bottom of the well. The differentiation medium was exchanged every 3 days. Raft cultures were harvested 10 to 14 days after induction of differentiation, formalin fixed, paraffin embedded, and stained by means of standard procedures.

G0/G1, S PHASE, AND G2/M ALTERATIONS BY FLOW CYTOMETRY

Ethanol-fixed cells were incubated with propidium iodide (20 µg/mL) and ribonuclease (200 µg/mL) for 20 minutes at 37°C. Cell cycle distribution was measured by a flow cytometer (FACScan; Becton Dickinson, Bedford, Mass) equipped with an air-cooled argon ion laser. At least 10000 events per sample were analyzed. ModFit LT (Verity Software House Inc, Topsham, Me) cytologic software program was used for data analysis. ModFit LT uses mathematical models to fit data from FACScan to generate curves of each cell cycle phase and area under the curve. Mean peak fluorescence was determined for each histogram. All analyses were performed in triplicate.

WESTERN BLOT ASSAY OF CELL CYCLE REGULATORY PROTEINS

Total cell lysates were prepared by incubating the cells in RIPA buffer (150-mmol/L sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.4% sodium dodecyl sulfate, 20-mmol/L EDTA, and 50-mmol/L Tris, pH 7.4) for 1 hour at 4°C. Equal amounts of protein from each sample were subjected to 7% to 14% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (BioRad, Hercules, Calif). The membrane was blocked with Blotto-Tween (10% nonfat milk, 0.05% Tween 20, 0.9% sodium chloride, and 50-mmol/L Tris, pH 7.5) and incubated with the primary antibodies against p16, p16β, p21, cyclin D1, MDM2, CDK4, and p53). A secondary antibody, horseradish peroxidase–conjugated IgG, was incubated with membranes and developed according to Amersham's enhanced chemiluminescence protocol (ECL; Amersham, Piscataway, NJ).

VEGF ANALYSIS

The concentration of VEGF was determined by means of an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minn). Cells (300000) were placed in 6-well plates for each experiment in a final volume of 3 mL of culture medium. During logarithmic growth, cells were analyzed at 24 and 48 hours after 70% to 80% confluent was reached. The supernatant (500 µL) of each well was then collected and stored in the refrigerator. Each sample was diluted 1:4 with the calibration diluent. Serial dilutions were prepared with the VEGF standard to yield an 8-point standard curve from 0 to 1000 pg/mL. The colorimetric enzyme-linked immunosorbent assay was performed in a 96-well plate. The intensity of the color developed was measured at a wavelength (λ) equal to 450 nm by means of a microplate reader (Molecular Devices, Sunnyvale, Calif), and a corrective reading, due to optical imperfections in the plate, was made at λ equal to 570 nm and subtracted from the reading at 450 nm. The data were analyzed by means of the Soft Max software program (Molecular Devices). The log of VEGF standard concentrations was graphed against the log of the optical density of experimental concentrations. Average concentrations of VEGF secreted by the cells were determined. All analyses were performed in triplicate.
a faster rate. Angiogenesis also promotes tumor cell metastasis by allowing access to nearby blood vessels and increasing vascular permeability. Vascular endothelial growth factor (VEGF) is a promoter of angiogenesis and plays a crucial role in up-regulating neovascularization, in endothelial proliferation, and in increasing vascular permeability. Vascular endothelial growth factor has been found to be overexpressed in advanced HNSCC and enhances its progression.

The development of in vitro progression models of the upper aerodigestive tract has been limited. Normal oral mucosal cells have been immortalized with human papillomavirus (HPV) 16,10 Immortal human gingival keratinocyte (IHGK) is an oral keratinocyte cell line that has been immortalized with HPV and has features that suggest that it is preneoplastic.10,11 We transformed the IHGK cells with a tobacco carcinogen (4-[methylnitrosamino]-1-[3-pyridyl]-1-butanone [NNK]). We examined alterations in angiogenesis and cell cycle regulation in this in vitro model of head and neck tumor progression to determine if it reflects alterations observed in patients with HNSCC.

In monolayer cultures, the morphologic characteristics of IHGK cells appeared similar to those of normal upper aerodigestive tract keratinocytes (flat or cuboidal) and the cells grew only in enriched medium (DK-SFM) without serum. DK-SFM is an enriched keratinocyte media. IHKG cells do not tolerate serum because of the high calcium concentration. After exposure to NNK, the derived IHGKN cells proliferated in growth medium (DMEM and 10% FCS) normally used for HNSCC cells. IHGK cells did not grow in DMEM/FCS. In an enriched, serum-free growth medium, the IHGK cells proliferated faster than IHGKN, HN30, and HN12 cells because DK-SFM is optimized to allow growth of IHGK cells (Figure 2).

Raft cultures of these cells were established, and sections of the 3-dimensional organoid were prepared. IHGK cells formed severe squamous dysplastic epithelium on raft culture (Figure 3A). IHGK cells were previously found not to be tumorigenic.10 IHGK cells displayed pleomorphic nuclei and cytoplasm along with keratin pearls were also observed. HN12 cells (invasive and poorly differentiated cancer) formed pleomorphic nuclei and cytoplasm with 1 to 2 cell layers and no defined basal cell layer (Figure 3B). Multiple areas of invasion into collagen along with keratin pearls were also observed. HN12 cells (invasive and poorly differentiated cancer) formed pleomorphic nuclei and cytoplasm with 1 to 2 cell layers and no defined basal cell layer (Figure 3E).

The distribution of cells in the G0/G1 phase was similar between IHGK and IHGKN cells: 48.9% vs 53.7% (Table 1). The distribution of IHGK cells (38.5%) in the S phase was greater than that of IHGKN cells (28.5%). However, IHGKN cells had a greater percentage of cells in G2/M (17.8% vs 12.7%). Expression of cell cycle regulatory proteins was not significantly altered between IHGK and IHGKN. The expression of p16 and p21 was minimally decreased (Figure 4) in IHGKN. The other cell cycle inhibitors and promoters of the cell cycle are all expressed at basal levels in IHGK and IHGKN cells (Figure 4). HN30 and HN12 did not express p16 or p16β. These HN cell lines have either a mutation or a homozygous deletion in the p16 gene. No difference in p53 expression was observed in IHGK and IHGKN. The expression of p53 was increased in HN30, which may represent altered processing since p53 is not mutated in HN30. HN12 had a lower level of p53 and p21 expression because of a known p53 mutation.12
The secretion of VEGF is an indicator of angiogenesis and was measured (Figure 5). IHGK cells produced less VEGF on day 1 than IHGKN (50.6 vs 245.6 pg/mL), along with a lower overall production rate (79% vs 133%; Table 2). IHGKN cells (245.6 pg/mL) produced more VEGF than HN12 (176.9 pg/mL) and HN30 (72.9 pg/mL) cells. The rate of increase in the IHGKN cells was comparable with that of head and neck cancer cells.

Using this in vitro model of head and neck tumor progression, we identified alterations in regulatory molecules associated with cell cycle regulation and angiogenesis. Since cultured normal oral keratinocytes have a limited life span (4-6 passages), their prolonged use in the laboratory is not possible. Cell lines have been developed from preneoplastic tissue and by immortalizing oral keratinocytes. Preneoplastic cell lines are not readily available. Normal mucosal cells have been immortalized with HPV16 to form IHGK cells. IHGK cells have features that suggest that they are preneoplastic. They are not tumorigenic in nude mice, do not proliferate in media in which HNSCC cells grow, do not exhibit anchorage-independent growth, and display preneoplastic (dysplasia) histological features on raft cultures. IHGK cells undergo spontaneous transformation when passed by demonstrating progressive chromosomal alterations and increasing in aneuploid population. Furthermore, the p53 gene is mutated in later passages. Therefore, transformation of IHGK occurs both with carcinogen transformation and spontaneously with serial passages. This model uses both tobacco carcinogen and HPV, which have been linked to the development of HNSCC. We have shown tumor progression by histological criteria (raft culture), loss of cell cycle regulation, and increased VEGF production.

The loss of G1 regulation in this model profiles that of HNSCC. In patients with HNSCC, Rb expression is not commonly (10%) lost, whereas p16 expression is frequently (80%) absent and cyclin D1 is overexpressed (38% to 49%). The loss of G1 cell cycle control

Table 1. Cell Cycle Phase in Head and Neck Cancer in an In Vitro Model

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Phase, %</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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</thead>
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<tr>
<td>IHGK</td>
<td>48.9</td>
<td>38.5</td>
<td>12.7</td>
<td></td>
</tr>
<tr>
<td>IHGKN</td>
<td>53.7</td>
<td>28.5</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td>HN30</td>
<td>78.5</td>
<td>14.4</td>
<td>7.1</td>
<td></td>
</tr>
<tr>
<td>HN12</td>
<td>55.4</td>
<td>22.5</td>
<td>21.8</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Raft organotypic culture. A, IHGK cells (severe squamous dysplasia). B, IHGKN cells (invasive and poorly differentiated cancer). C, Normal mucosal cells (unrelated patient). D, HN30 cells (invasive and well-differentiated cancer). E, HN12 cells (invasive and poorly differentiated cancer). SCCa indicates squamous cell carcinoma.

Figure 4. Expression of cell cycle regulators by Western blot analysis showing the expression of p16, p53, p21, p16β, cyclin D (CD) 1, MDM2, and CDK2 in IHGK, IHGKN, HN12, and HN30 cells.
takes place early in HNSCC development, since p16 expression is lost in 43% of dysplastic lesions\(^3,21\) and 80% of tumor specimens. The p16 gene is functionally deleted by homozygous deletion, promoter methylation, or mutation.\(^3,22\) Since the HPV-E7 gene product inactivates Rb, the key regulatory event in the p16-CDK4-Rb regulatory pathway is Rb inactivation through E7 in this model and not by p16 deletion or cyclin D1 overexpression.

Loss of CDK2 inhibition occurs by the loss of p53 or p16\(^b\) in patients with HNSCC.\(^2\) The p16\(^b\) gene product promotes MDM2 degradation.\(^21\) Since MDM2 increases degradation of p53, p16\(^b\) promotes the inhibitory effect of p53 on the cell cycle.\(^21\) The p53 gene causes G\(_1\) cell cycle arrest and apoptosis and regulates transcription. In preneoplastic cell cultures, alterations in cell cycle promoters and inhibitors have not been delineated. In this model, the p53 protein in both IHGK and IHGKN cells is inactivated by E6. Since IHGK cells develop spontaneous p53 mutation in later passages, cells at less than 100 passages were used. Therefore, the inactivation of G\(_1\) in this model occurred at the immortalization (early) step with the transfection of HPV. No further loss of cell cycle regulation was observed after preneoplastic cells (IHGK) were exposed to a carcinogen.

The formation of new blood vessels, angiogenesis, in the tumor microenvironment causes rapid tumor growth through the increased oxygen and nutrient supply. The balance of positive and negative regulators of endothelial cell proliferation can lead to angiogenesis. Vascular endothelial growth factor is a promoter and a marker of angiogenesis that causes increased vascular permeability and endothelial cell growth, migration, and differentiation.\(^4,5\) The overexpression of VEGF is crucial in the progression of many cancers, including HNSCC.\(^6,7\) Increased expression of VEGF occurs late in tumor progression and correlates with the ability of tumors to grow aggressively and metastasize. Since VEGF is produced at a higher rate in carcinoma cell lines (HN12, HN30, and IHGKN) as opposed to preneoplastic cells (IHGK), this model simulates a late step in head and neck tumor progression that is observed in patients.\(^6,7\) Although our in vitro system demonstrated production of VEGF (angiogenesis promoter), an in vivo system that demonstrates new endothelial cell proliferation and blood vessel production needs to be further examined before this model may be used to test antiangiogenic effects of chemotherapy and novel inhibitors of angiogenesis.

**CONCLUSIONS**

We have characterized alterations in angiogenesis and cell cycle regulation in a model of head and neck tumor progression and found these alterations to be similar to changes in HNSCC cell lines. Transformation of IHGK cells resulted in the activation of VEGF associated with angiogenesis. Inactivation of the G\(_1\) cell cycle regulation occurs before transformation and is sustained after carcinogen exposure. Many other pathways that need to be further delineated are involved in the transformation of IHGK to IHGKN cells. After this model is tested against other preneoplastic and cancerous cells, it can be used to gain an understanding of the molecular progression of HNSCC and to test novel therapeutic and preventive approaches.

Accepted for publication April 11, 2000.

This work was supported by grants IN-162 and CRTG-99-246-01-CCE from the American Cancer Society, Atlanta, Ga.

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### REFERENCES


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#### Table 2. VEGF Secretion in IHGK, IHGKN, HN30, and HN12 Cells*

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Day 1</th>
<th>Day 2</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHGK</td>
<td>50.6</td>
<td>90.8</td>
<td>79</td>
</tr>
<tr>
<td>IHGKN</td>
<td>245.6</td>
<td>573.4</td>
<td>123</td>
</tr>
<tr>
<td>HN30</td>
<td>72.9</td>
<td>306.6</td>
<td>321</td>
</tr>
<tr>
<td>HN12</td>
<td>176.9</td>
<td>609.5</td>
<td>245</td>
</tr>
</tbody>
</table>

*VEGF indicates vascular endothelial growth factor.*


