Cyclin D1 Expression in Squamous Cell Carcinomas of the Head and Neck and in Oral Mucosa in Relation to Proliferation and Apoptosis

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ABSTRACT

Deregulation of expression of the cell cycle regulator cyclin D1 (cD1) may be responsible for rapid proliferation of squamous cell carcinoma of the head and neck (SCCHN). We have studied the expression of cD1 in 46 SCCHNs using immunohistochemistry. Before biopsy, the patients received an in vivo infusion of iododeoxyuridine (IdUrd) for cell proliferation assessment. Additionally, the level of apoptosis was estimated using in situ end labeling (ISEL). Among 33 tumors, the proportion of cD1+ cells varied from 0.5 to 51.3% (19.9 ± 2.2%). Thirteen tumors did not express cD1. The fraction of S-phase (IdUrd-positive) cells was 26.3 ± 1.8% in cD1+ versus 20.0 ± 2.4% in cD1− tumors (P = 0.06). The percentages of cD1+ cells and of S-phase cells were not correlated (P = 0.37). Apoptosis was detected by ISEL in 15 of 33 tumors studied. ISEL-positive tumors contained a significantly higher proportion of cD1+ cells (14.9 ± 2.6%) than cD1− ones (7.9 ± 2.8%; P = 0.03). There was a positive correlation between the percentage of cD1+ cells and the degree of ISEL (r = 0.54; P < 0.001). In noninvolved oral mucosa, cD1+ cells were located primarily in the suprabasal layers (29.3 ± 3.8% versus 1.2 ± 0.2% in the basal layer). Only 23 of 44 mucosal specimens contained cD1+ cells. All cD1− samples were proliferatively active and contained IdUrd-labeled cells. The percentage of cD1+ cells in the oral epithelium from nontumor controls (uvula samples) was significantly higher than in the SCCHN group in both basal (2.4 ± 0.4%; P = 0.008) and suprabasal (42.7 ± 3.3%; P = 0.005) layers. Additionally, whereas in uvuli, cD1+ cells were distributed evenly along the epithelial lining, in SCCHN samples the regions showing cD1 expression alternated with areas in which cD1 expression was undetectable. These data indicate that cD1 expression in SCCHN varies among tumors and is not correlated with cell proliferation. In noninvolved oral mucosa, cD1 expression differs from that in truly normal epithelium obtained from nontumor patients. A correlation between cD1 expression and the extent of ISEL positivity suggests a possible involvement of cD1 expression in the apoptotic pathways.

INTRODUCTION

D-type cyclins and the CDKs3 play important roles in cell cycle regulation (1). Their primary target is a checkpoint in late G1, also known as a "restriction point" (2). At this point, the cell commits itself to DNA synthesis and subsequent division. In mammalian cells, complexes of D-type cyclins and CDKs, primarily CDK4 and CDK6, induce phosphorylation of pRb, releasing a cassette of transcription factors and other cell cycle-related proteins from the inhibitory control of pRb. The cyclin D family consists of three proteins (D1, D2, and D3). They are in part tissue specific, with most cell types expressing D3 and either D1 or D2 (3). Among the three D-type cyclins, cD1 has been best characterized as an oncogene. This protein is encoded by CCND1 gene on chromosome 11q13 (4). cD1 is deregulated in many types of human malignancies (5). Overexpression of cD1 may be a result of clonal gene rearrangement by translocation. This mechanism was described for some parathyroid adenomas in which CCND1 comes under the control of parathyroid hormone gene promoter (6) and in centroblastic B-cell lymphomas in which the expression of cD1 is brought under the control of immunoglobulin heavy chain enhancer (7). In many human epithelial tumors, cD1 is overexpressed as a result of amplification of the 11q13 region (4). cD1 is deregulated in 30–65% of SCCHNs (8–13) and in 15–40% of breast carcinomas (5, 14, 15). Overexpression or ectopic expression of cD1 may result in the loss of cell-cycle control with consequent increased tumor cell proliferation.

Additionally, several experimental observations suggest a direct role of cD1 in neoplastic transformation. In cooperation with ras, cD1 induced transformation of rat embryo fibroblasts (16). cD1 also cooperates with Myc in the generation of lymphomas in transgenic mice (17). Under the control of the mouse

1 The abbreviations used are: CDK, cyclin-dependent kinase; cD1, cyclin D1; IdUrd, iododeoxyuridine; ISEL, in situ end labeling; LI, labeling index; SCCHN, squamous cell carcinoma of the head and neck; pRb, retinoblastoma protein; NCI, National Cancer Institute; DAB, 3,3′-diaminobenzidine.

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mammary tumor virus long-terminal repeat, the overexpression of cDL results in the development of mammary hyperplasia and carcinomas in transgenic mice (18). cDL acts synergistically with ABL oncogenes in transforming fibroblasts and hemopoietic cells in culture (19).

Although most studies concentrate on the role of cDL as a positive regulator of the cell cycle through phosphorylation of the pRb family, several reports indicate that cDL may also play other roles in the life cycle of the cell. Thus, nonproliferating senescent human diploid fibroblasts that have reached the end of their *in vitro* lifespan express 5-fold higher levels of cDL than actively dividing early passage cells (20, 21). Additionally, the selective induction of cDL was observed in postmitotic neurons during apoptosis (22). Taken together, these observations suggest that cDL plays an important role in the network regulation of many cell life cycle events.

SCCHNs represent a biologically and clinically heterogeneous group of tumors. Several studies have repeatedly confirmed the very high rate of cell proliferation in a high proportion of these tumors (23–25). Cell proliferation rates correlate with patient survival (26–28). Preliminary results of clinical studies suggest that cell proliferation parameters may provide useful information for scheduling radiation therapy regimens in these patients (26, 27). The rapid proliferation of SCCHN, with potential doubling time ranging from only 2 to 4 days (23–26), may in part result from molecular abnormalities in these tumors. The *CCND1* gene amplification (8–11) and overexpression of cDL on the protein level (12, 13) in SCCHN may be responsible for a permanent positive stimulation of proliferation in these tumors.

As part of an ongoing investigation of genes involved in cell proliferation in SCCHN, we have examined the expression of cDL in surgically excised tumors, in adjacent noninvolved epithelium, and in oral epithelium samples obtained from non-tumors. Several studies have repeatedly confirmed the very high rate of cell proliferation in a high proportion of these tumors (23–25). Cell proliferation rates correlate with patient survival (26–28). Preliminary results of clinical studies suggest that cell proliferation parameters may provide useful information for scheduling radiation therapy regimens in these patients (26, 27). The rapid proliferation of SCCHN, with potential doubling time ranging from only 2 to 4 days (23–26), may in part result from molecular abnormalities in these tumors. The *CCND1* gene amplification (8–11) and overexpression of cDL on the protein level (12, 13) in SCCHN may be responsible for a permanent positive stimulation of proliferation in these tumors.

As part of an ongoing investigation of genes involved in cell proliferation in SCCHN, we have examined the expression of cDL in surgically excised tumors, in adjacent noninvolved epithelium, and in oral epithelium samples obtained from non-tumor patients. Additionally, the relationships of cDL expression, cell proliferation, and the level of apoptosis have been addressed in this study.

**MATERIALS AND METHODS**

**Patients.** Forty-six SCCHN patients were studied. Thirty-six were infused with IdUrd for cell proliferation studies. The IdUrd infusion protocol was reviewed and approved by the Institutional Review Board of Rush University, NCI, and the Food and Drug Administration. IdUrd and BrdUrd were supplied by the NCI. Informed consent required by the local Institutional Review Board, NCI, and the Food and Drug Administration was obtained from every patient before the administration of IdUrd.

Table 1 provides the clinical information for the patients and tumors that were studied. IdUrd infusions were performed between 8 a.m. and 12 p.m., when the circadian DNA synthesis peak in oral epithelia occurs (29). IdUrd (100 mg/m²) was given i.v. over 30 min.

Forty-four specimens of noninvolved, normal-appearing mucosa were also obtained during the tumor biopsy. As a nontumor “control” for cDL studies, uvula specimens from 14 nonsmoking persons were obtained during minor surgical procedures for snoring. Additionally, six tonsillar samples were used as nontumor controls.

**Staining for cDL.** Slides with deparaffinized sections were placed in 10 mM citrate buffer and microwaved for 18 min. The slides then were washed in PBS and stained by a Ventana automated immunohistochemical stainer (Ventana Medical System, Tucson, AZ). Primary monoclonal anti-cDL antibody (DCS-6, Novocastra, Newcastle upon Tyne, United Kingdom) was used for formalin-fixed paraffin-embedded tissues. For apoptosis studies, the tumor biopsies were fixed in Bouin’s solution, dehydrated in methanol, and embedded in glycol methacrylate. Sections (2–3 μm) were placed on alcian blue-coated coverslips and used for ISEL studies.

**Assessment of Proliferation.** Detailed cell cycle kinetic studies in SCCHN have been reported elsewhere (30, 25). In the present studies, S-phase cells were stained with an IdUrd/BrdUrd-specific monoclonal antibody, 3D9 (a gift from Dr. G. L. Meyers, Roswell Park Memorial Institute, Buffalo, NY). Briefly, deparaffinized sections were treated sequentially with 3% H₂O₂, Pronase, 4 N HCl, 3D9 antibody, the avidin-biotinperoxidase conjugate (Vestacastain Elite ABC Kit; Vector Laboratories, Burlingame, CA), and DAB. Finally, the sections were counterstained with hematoxylin. The LI was determined in several randomly selected fields of the section. At least 500–1000 tumor cells were counted in every field. Four to six fields (2000–5000 tumor cells) representing areas with high and low proportions of labeled cells were studied to calculate the average values of LI for each biopsy.

**Assessment of Apoptosis by ISEL of DNA.** ISEL was carried out on glycol methacrylate-embedded sections as detailed elsewhere (31). Briefly, after pretreatment with sodium...
chloride-sodium citrate solution at 80°C and with 0.5% pepsin (Sigma Chemical Co., St. Louis, MO) in HCl (pH 2) at 37°C, the sections were incubated with a mixture of dATP, dCTP, dGTP (0.01 M, Promega, Madison, WI), biotinylated dUTP (0.001 M, Sigma Chemical Co.), and Escherichia coli DNA polymerase I (20 units/ml, Promega) at 18°C. Incorporation of biotinylated dUTP was visualized using avidin-biotin-peroxidase conjugate (Vectastain Elite ABC Kit) and DAB. Thus, cells labeled positively for ISEL showed brown staining in their nuclei under light microscopy.

A subjective rating scale was formulated to measure the degree of ISEL positivity: 0, undetectable; 1+, few ISEL-positive cells in nontumor compartments; 2+, up to 5% ISEL-positive tumor cells; 3+, 5–15% ISEL-positive cells; 4+ to 5+, 15–35% ISEL-positive cells; and 6+ to 8+, >35% ISEL-positive cells. Before the scoring of the ISEL-stained slide, the adjacent section was stained with H&E, and a distinction between tumor and nontumor compartments was made by three independent observers, including experienced pathologists. Scoring was performed by consensus by two observers.

In 13 of these 33 specimens, we counted the percentage of apoptotic bodies in biopsies as well. The apoptotic body count and the degree of ISEL were well correlated with each other (r = 0.586; P = 0.045).

Statistical Analysis. The Mann-Whitney U test and Spearman correlation analysis were performed using SPSS for Windows (standard package).

**RESULTS**

cD1 Expression and Distribution in Tumor Tissue. Thirty-three tumors contained cD1+ cells varying in numbers and intensity of staining. In 13 tumors, cD1+ cells were undetectable. Positive staining was limited to cell nuclei. Fig. 1, a–c, shows different patterns of cD1 positivity in tumor tissue. In some specimens, cD1+ cells were scattered along the whole tumor section. In other biopsies, only limited areas of the tumor contained cD1+ cells. Occasionally, cD1+ nuclei of nontumor cells were present in the interstitial tissue surrounding tumor islands.

There was a high degree of correlation between the results of quantitative and semiquantitative analyses (r = 0.901; P < 0.0001). The proportion of cD1+ tumor cells (cD1-negative cases are not included in these calculations) varied within the range 0.5–51.3% (19.9 ± 2.2%; mean ± SE). Fig. 2 illustrates the frequency distribution of tumors with different expression of cD1. There was no statistically significant difference between primary and relapse patients (15.2 ± 3.1% and 13.1 ± 2.8%, respectively; P = 0.61). On average, the proportion of cD1+ was higher in moderately and poorly differentiated tumors than in well-differentiated ones (Table 2). However, the differences were not significant (P > 0.05). Similarly, a slight difference was observed between the groups of stage I and II patients (9.5 ± 3.8%) and stage III and IV patients (16.2 ± 2.8%), which did not attain statistical significance (P = 0.20).

cD1 Expression and Cell Proliferation. In the group of cD1-positive tumors, parallel cD1 and LI studies were performed in 26 samples. The percentage of S-phase cells labeled with IdUrd (26.3 ± 1.8%; range, 12.1–51.5%) was higher than the proportion of cD1+ cells (18.0 ± 2.6%; P = 0.008). There was no correlation between the percentage of cD1+ cells and LI in cD1-positive tumors (r = 0.185; P = 0.375). All cD1-negative tumors contained varying proportions of S-phase cells (7.4–33.5%; on average, 20.0 ± 2.4%). The difference between cD1-negative and cD1-positive tumor LIs was not significant (P = 0.06).
cD1 and Apoptosis. ISEL was performed in 33 SCCHNs. In 18 of 33 tumors studied, apoptotic cells were undetectable by ISEL. ISEL-negative tumors contained significantly lower proportions of cD1+ cells (7.9 ± 2.8%) than did ISEL-positive tumors (14.9 ± 2.6%; P = 0.03). There was a positive correlation between the percentage of cD1+ cells and the degree of ISEL (r = 0.538; P < 0.001). Interestingly, the LI values in these two groups of tumors were practically the same (21.4 ± 2.8% and 23.0 ± 2.4%, respectively). Thus, the degree of apoptosis measured by ISEL correlates with cD1 expression but not with LI.

cD1 in Normal Oral Epithelium. cD1+ cells in the epithelium from nontumor controls (uvula) were distributed evenly along the whole epithelial lining. The intensity of nuclear staining was weak to moderate and never reached the high intensity level frequently present in tumor cells. On the other hand, the proportion of cD1+ cells was very high in all uvula specimens (Table 3; Fig. 3). The proportion of cD1+ cells was especially high in suprabasal layers in which, on average, every other cell expressed cD1 (Table 3). In contrast, only 2.4 ± 0.4% of cells in the basal layer were cD1+.

In noninvolved oral mucosa from SCCHN patients, cD1 positivity patterns differed from those present in normal mucosa (uvula) in the following ways: (a) only 23 out of 44 available specimens contained cD1+ cells. Ten specimens were cD1 negative, and in 11 samples, the intensity of staining was too low to count labeled cells. cD1-negative samples, however, always contained IdUrd-labeled cells; (b) cD1 positivity appeared in a "patchy" pattern when regions showing cD1 expression alternated with areas in which cD1 was undetectable; and (c) the proportion of cD1+ cells counted in "cD1-positive areas" was significantly lower than in uvula in both basal and suprabasal layers (Table 3). There was no correlation between the percentage of cD1+ cells and the LI values in both basal and suprabasal layers (P > 0.05).

"Normal" mucosa samples from SCCHN patients were ISEL negative (ISEL was not performed on uvula samples).

**Table 2**

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>n</th>
<th>Mean ± SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well</td>
<td>7</td>
<td>6.3 ± 2.6</td>
<td>0–17.0</td>
</tr>
<tr>
<td>Moderate</td>
<td>28</td>
<td>15.7 ± 2.7</td>
<td>0–51.3</td>
</tr>
<tr>
<td>Poor</td>
<td>11</td>
<td>15.5 ± 5.1</td>
<td>0–48.3</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Epithelial layer</th>
<th>Uvula (n = 14)</th>
<th>Noninvolved epithelium from SCCHN patients (n = 23)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>2.4 ± 0.4 (0.3–4.7)</td>
<td>1.2 ± 0.2 (0–4.9)</td>
<td>0.008</td>
</tr>
<tr>
<td>Suprabasal</td>
<td>42.7 ± 3.3 (29.4–67.7)</td>
<td>29.3 ± 3.8 (2.5–52.7)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Our study demonstrates that the expression of cD1 in SCCHN is highly variable. This is in agreement with the results of Bartkova et al. (12), who studied cD1 expression in 52 SCCHNs. They used the same DCS-6 monoclonal antibody as we did, but they used methacarn-fixed instead of formalin-fixed specimens. Our study demonstrates that distinct and reproducible staining may be achieved on formalin-fixed biopsies using the microwave technique. This is an important finding, because it makes it possible to study large series of SCCHN on archival specimens.

To our knowledge, this is the first study that compares cD1 expression and the percentage of S-phase cells in SCCHN. Because both parameters are related directly to cell proliferation, it would be logical to expect that the proportion of cD1-positive cells would be highly correlated with the percentage of S-phase cells (LI). However, we have not found any correlation...
between these two parameters. This observation is in agreement with the data demonstrating the involvement of cD1 into the processes other than cell proliferation (16–22).

Thirteen of 46 SCCHNs (30%) in this study did not express cD1 despite repeated staining sessions. The absence of cD1 expression was not due to the low proliferation rate of the tumors, because all 13 tumors demonstrated extensive IdUrd labeling (LI varied from 7.4 to 36.4%). Unidentified proportions of cD1-negative tumors have also been reported previously in studies of SCCHN (12) and breast carcinoma (32). Immunohistochemistry alone is not sufficient to answer the question whether these “cD1-negative” tumors are really lacking cD1 activity or a technical problem is present. DNA analysis (Southern blot) has demonstrated the presence of cD1 gene in 100% of SCCHNs studied (10). We did not find any reports on cD1 mRNA analysis in SCCHNs. In the breast, carcinoma cD1 mRNA was present in all of the tumors in the series as shown by in situ hybridization (33). Bartkova et al. (5) used immunoblotting to detect cD1 in 23 tumors of various origins that had different degrees of cD1 positivity by immunohistochemistry. It was demonstrated that weakly stained tumors were cD1 negative by immunoblotting, indicating a higher sensitivity for immunohistochemistry. Nevertheless, the question of the presence of cD1 protein still remains to be studied in SCCHN, preferably with a complex of methods including immunohistochemistry, Western blot, and immunoprecipitation. On the other hand, in those SCCHNs that are cD1 negative, proliferation may be driven by other molecules from the cyclin D family. Another possibility is that the function of Rb, the target molecule for cD1, may be lost in these tumors through mutations or by other mechanisms (34), making the expression of cD1 unnecessary for cell proliferation. The deletion of Rb gene, however, is not a common finding in SCCHN, even in cases involving 13q deletions (35). Clearly, the problem of cD1 “negativity” of SCCHN remains to be investigated. Nevertheless, one of the practical conclusions from these observations is that cD1 expression by immunohistochemistry cannot be used as a surrogate proliferation marker.

The positive correlation between cD1 expression and the degree of apoptosis is an unexpected finding. ISEL methodology enables visualization of cells in the early stages of apoptosis before morphologically recognizable apoptotic bodies are organized (31). In the study described here, we failed to demonstrate ISEL positivity in noninvolved epithelium from SCCHN patients. These observations may indicate that apoptosis may not be an essential mechanism in the regulation of cell renewal in oral epithelium in SCCHN patients. However, the role of apoptosis in truly normal mucosa still remains to be established.

Most tumors either were ISEL negative or showed very low levels of apoptosis. On the other hand, 15 of 33 tumors (45%) in this study demonstrated various rates of ISEL positivity. The association of higher levels of cD1 expression in this group of tumors was not correlated with cell proliferation as evidenced by L1. Hence, increased apoptosis does not appear to be a tumor response to offset an increased cell proliferation.

Although the involvement of cD1 in cell proliferation is well established, the association of cD1 with apoptosis still remains poorly explored. Increased cD1 expression is associated with apoptosis in explanted rat postmitotic neurons (22). Additionally, in mouse mammary epithelial cells, an induced over-expression of cD1 increases susceptibility to induction of apoptosis (36). The observations described here may thus represent another contribution to this problem. The association of cD1 expression with apoptosis may be a secondary event of a deregulated proliferation/apoptosis gene interplay. Products of many genes are involved in the network regulating these two phenomena. For example, the role of p53 in the initiation of apoptosis is well established (37, 38). In different cell systems, p53 may either inhibit (39) or induce (40) cD1 via the same p21 pathway. Another oncoprotein, c-Myc, cooperates with cD1 during induction of proliferation (41, 42). On the other hand, c-Myc is implicated in apoptotic responses and cooperates with p53 to induce apoptosis (43, 44). There are many other examples of multifunctionality of proliferation/apoptosis-associated molecules, many of which are implicated into oncogenesis. In light of these observations, it seems possible that cD1 may be involved directly in some presently unknown apoptotic pathways.

In normal epithelium from nontumor patients, cD1+ cells were distributed evenly along the epithelial lining without cD1-negative areas. The intensity of cD1 immunostaining is not as strong as in some of the tumor samples, which is in accord with the data of Bartkova et al. (12). However, the percentage of cD1+ cells is very high. In some specimens, two of every three cells are cD1 positive. The high frequency of cD1+ cells in normal oral epithelium may be tissue specific. According to Sherr et al. (45), cD1 is induced early in G1 and may be expressed throughout the cell cycle as long as a growth factor is present.

In the epithelium from SCCHN patients, both the distribution and the frequency of cD1+ cells differed from those of truly normal epithelium. In 10 specimens, cD1 was undetectable, but IdUrd-labeled cells were present. In 11 specimens, the staining was so weak that it was impossible to distinguish between many labeled and unlabeled cells. In the remaining 23 samples, cD1+ cells were found in only limited regions of the epithelial lining, with large areas being cD1 negative. The percentage of cD1+ cells in these specimens (counted in cD1-positive regions only) was significantly lower than in unvli. These abnormalities in cD1 expression may be a result of either local disturbances produced by neighboring tumor (circulatory insufficiency, direct effects of tumor-originating substances, and others) or of field cancerization (46, 47).

In both truly normal and tumor-associated mucosa, cD1-positive cells are located mainly in suprabasal layers of oral epithelium. The frequency of cD1+ cells in the basal layer is very low. S-phase cell distribution has the same pattern. These observations confirm our earlier data that only cells in suprabasal layers are proliferatively active in human oral epithelium from SCCHN patients (48, 49).

We conclude the following: (a) the expression of cD1 in SCCHN varies over a wide range; (b) about 30% of actively proliferating tumors are cD1 negative by immunohistochemistry; (c) cD1 expression correlates with the level of apoptosis in SCCHN; and (d) in normally appearing oral mucosa from SCCHN patients, cD1 expression is severely deregulated compared to truly normal control.
REFERENCES


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