Markers of Cell Proliferation in Normal Epithelia and Dysplastic Leukoplakias of the Oral Cavity

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Abstract
The expression of several markers of epithelial cell proliferation was analyzed to establish baseline data for future chemoprevention studies of oral premalignant lesions. Punch biopsies (n = 60) from three different sites of oral mucosa (bucca, lateral tongue, and the floor of the mouth) were obtained from 20 normal donors of both sexes. After formaldehyde fixation and paraffin embedding, immunohistochemistry was used to detect the proliferation markers Mib-1, cyclin D1, and centromere-associated protein CENP-F. Analysis of sections stained for the three markers showed similar patterns, i.e., a low labeling index (LI) in the basal layer and a high LI in the parabasal layer at all three intraoral sites. No proliferative activity was seen above the parabasal layer (superficial layer). All sites showed similar Mib-1 LI values for the proliferative markers. The tongue epithelium exhibited higher parabasal LIs of cyclin D1 and CENP-F than did the other two sites. No significant differences were detected between smokers and nonsmokers. The data from normal mucosa were compared with those from low (n = 30) and high (n = 17)-grade dysplastic leukoplakias. The Mib-1 LI showed a very significant change, with a 9-fold increase in the basal layer LI in dysplastic leukoplakias. Cyclin D1 and CENP-F showed similar trends with increments of up to 7-fold in the basal layer of high-grade dysplasia.

Although the proliferative activity of the parabasal layer was similar in normal and leukoplakic epithelia, the superficial layer showed a significant increment in proliferative activity mainly in high-grade leukoplakia. These studies suggest that proliferation markers in the basal and superficial cells of premalignant lesions may serve as surrogate end point biomarkers for chemoprevention trials.

Introduction
Leukoplakia is the most common premalignant lesion of the oral mucosa. This lesion is defined as a white patch or plaque that cannot be diagnosed as any other disease and is not associated with any mechanical or chemical irritant except for the use of tobacco (1, 2). Histologically, the lesions are subclassified according to the degree of dysplasia and growth rate changes (2, 3). Because premalignant lesions of different anatomical sites are characterized by increased cell proliferation (4–6) that usually parallels the degree of dysplasia, cell proliferation markers may be used to assess the type and degree of oral premalignant lesions. Thus, immunohistochemically detectable proliferation markers could be of great value in predicting lesion behavior and may serve as surrogate endpoint biomarkers in cancer chemoprevention studies to evaluate possible regression or improvement in abnormal features in the tissues of subjects at increased risk. The assessment of surrogate end point biomarkers is possible because invasive epithelial neoplasms are known to be preceded by intraepithelial proliferations with a spectrum of cellular abnormalities extending from mild dysplasia to carcinoma in situ (7).

Oral leukoplakia with dysplasia is a well-recognized precursor of invasive SCC of the oral cavity. The percentage of leukoplakic lesions that progress to invasive SCC is directly related to the severity of the dysplasia, ranging from under 5% for leukoplakias with mild to moderate dysplasia to as high as 43% for leukoplakias containing severe dysplasia/carcinoma in situ (2). Changes in the proliferative capacity of premalignant oral lesions have been reported in the past (3, 8–14). Nevertheless, because of the variety of methods used to assess these changes and the relatively little concern for the baseline proliferative values of the oral epithelia at different intraoral sites, this study was designed to evaluate the proliferative characteristics of normal and leukoplakic epithelia using modern markers of cell proliferation.

We have selected three proliferative markers of precursor lesions of the oral mucosa based on recent work in specimens from several anatomical sites (6, 15–21). These markers are Ki-67, cyclin D1, and CENP-F. Ki-67 or Mib-1 is the marker used most frequently to demonstrate the percentage of proliferating cells in all non-G0 phases of the cell cycle (22). D-type cyclins, including cyclin D1, are the rate-limiting controllers of...
G1-phase progression in mammalian cells and are expressed by cells in the G1–S phases of the cell cycle (23). Amplification and overexpression of cyclin D1 have been identified in a high proportion of SCCs of the head and neck (19, 24–28). CENP-F is a human kinetochore protein that is prominently detected in the G2 and M phases of the cell cycle (29). Thus, this maybe a new cell cycle marker for identifying proliferating cells in G2-M phases.

To study the possible use of these biomarkers in future cancer chemoprevention trials, we have evaluated the proliferative behavior of normal oral mucosa from three different intraoral sites and of oral leukoplakias with different degrees of dysplasia using antibodies raised against Ki-67.

Materials and Methods

Tissue Samples. Normal oral epithelia from three different sites were examined in tissues obtained from 18 autopsies and 20 healthy donors. Three different sites (i.e., the floor of the mouth, lateral tongue, and bucca) were selected because they provided the most frequent sites of leukoplakias in our patient population. Similarly, 30 leukoplakia biopsies with mild to moderate dysplasia (LGD) and 17 leukoplakia biopsies with severe dysplasia or carcinomas in situ (HGD) were obtained. The tissues were fixed overnight in 10% phosphate-buffered formaldehyde and embedded in paraffin using a short embedding cycle (2.5 h). Paraffin sections (5 μm) were cut and placed on poly-L-lysine-coated slides for the immunohistochemical detection of proliferation markers Mib-1 (Ki-67), cyclin D1, and CENP-F by the immunoperoxidase technique. To serve as negative controls, sections were counterstained with hematoxylin and mounted.

Immunohistochemistry. Antigen retrieval was performed for either 5 min in sodium citrate (Mib-1) or 10 min in distilled water (cyclin D1 and CENP-F). The following primary antibodies were used: (a) Mib-1 to detect the Ki-67 antigen in human paraffin-embedded tissues (mouse monoclonal antibody; Immunotech, Westbrook, ME); (b) anti-cyclin D1 (mouse monoclonal antibody Ab-3; Calbiochem, Cambridge, MA); and (c) anti-CENP-F clone D10 (29). An avidin-biotinperoxidase kit (Vectastain Elite, Burlingame, CA) was used, followed by the chromagen 3,3'-diaminobenzidine to develop the immunostain. To evaluate the relative changes in CENP-F and cyclin D1, we used the tongue LIs for comparisons of normal and leukoplakic specimens.

L1 and Statistical Analysis. Positive nuclear immunostaining was evaluated in the basal, parabasal, and superficial epithelial layers by determining the percentage of positively stained cells (positive cells: total cells × 100), which constitutes the LI. The layers are defined as follows: (a) the basal layer is the single row of cells directly in contact with the basement membrane; (b) the parabasal layer is defined as two rows of cells immediately toward the surface of the basal epithelial cells; and (c) the superficial layer is composed of all of the cells present between the parabasal layer and the surface. Stained tissue sections were viewed under a light microscope (×40 objective; Zeiss, Oberkochen, Germany). Each biomarker was evaluated in the same areas on successive sections. A total of 200–800 cells/layers were counted in each specimen. A total of 75,000–89,000 cells/marker were counted. A semiquantitative scale ranging from 0 (no expression) to 3 (high expression) was used to select cells with high (a score of 3) and moderate expression (a score of 2; Ref. 30). Marginal expression (a score of 1) and no expression were disregarded. The LI was calculated as the percentage of positively stained cells (scores of 2 and 3) divided by the total number of cells counted per layer.

Kruskal-Wallis tests were used to test for differences between epithelial types (normal, leukoplakia, and carcinoma in situ) in the proportion of cells that stained positive for a particular marker of epithelial cell proliferation (Mib-1, cyclin D1, and the centromere-associated protein CENP-F). These analyses were performed separately by marker and layer (basal and parabasal layers). The marker data obtained on samples from the superficial layer were highly skewed, with a large number of samples having no positively stained cells. For this reason, the data for each marker from the superficial layer samples were grouped into two categories: (a) no positively stained cells; and (b) one or more positively stained cells. Fisher’s exact test was used to test for differences between epithelial types in the distribution of the discrete marker data. Pairwise comparisons between epithelial types were conducted when a significant overall association was detected between the expression of a marker and epithelial type.

A stepwise discriminant analysis was conducted to identify a subset of the marker data from the different layers that was useful for discriminating among epithelial types. The entry and elimination criteria for this variable reduction procedure were set at P = 0.05. Proliferation marker data from normal tissues were analyzed using the generalized estimating equations approach to binary outcomes (31) to test the effects of the following covariates: (a) intraoral sites (tongue, the floor of the mouth, bucca); (b) smoking status (current smoker, nonsmoker); and (c) age on the proportion of positively stained cells. Again, analyses were conducted separately by marker and layer. Robust estimates of the average were used for statistical inferences. These SE estimates account for any overdispersion or intraindividual dependence.

All analyses were conducted using the SAS statistical software package (SAS Institute, Inc., Cary, NC).

Results

Expression of Mib-1, Cyclin D1, and CENP-F in Normal Oral Mucosa. Expression of all three markers in normal epithelia was limited to the germinative layer, i.e., the basal and parabasal layers (Fig. 1). Cells in the superficial layer did not express proliferation markers. A comparison of the three markers showed that LIs of the autopsy-derived tissues were different from those of the biopsies. This was seen in all intraoral sites (Fig. 2). With the exception of the Mib-1 LIs of the basal layer in which there was no statistically significant difference between autopsy- and biopsy-derived tissues (Fig. 2), all of the other LIs were lower in the autopsy-derived specimens. In addition, cyclin D1 and CENP-F were almost undetectable in the tissues obtained at autopsy (Fig. 2).

Irrespective of the mode of tissue procurement, parabasal cells showed a higher LI than did basal epithelial cells. Because biopsy-derived material is a more reliable control for our leukoplakia study, we used these tissues for a more in-depth analysis. Age, gender, and smoking habits did not significantly influence Mib-1 and CENP-F results. Of the three markers, Mib-1 had the highest LI (Figs. 1 and 3). Although we noted no site-related Mib-1 differences, the tongue epithelium showed slightly higher cyclin D1 and CENP-F LIs than did epithelia from either the bucca or the floor of the mouth (Fig. 3). The CENP-F LI of the tongue basal layer and the cyclin D1 LI of the parabasal layer showed statistically significant increases when compared with other sites (P > 0.0001 and 0.004, respectively). To evaluate the relative changes in CENP-F and cyclin D1, we used the tongue LIs for comparisons of normal and leukoplakic
Fig. 1. Immunohistochemical detection of proliferative markers in normal oral epithelia. Mib-1 was seen in the basal and parabasal layers in the epithelia of the buccal mucosa (A), tongue (B), and floor of the mouth (C). Although some basal cells showed strong immunostaining, most of the intensely stained nuclei corresponded to the parabasal layer (arrowheads). Cyclin D1 immunohistochemistry of the three intraoral sites showed a very similar distribution of immunostaining. For example, tongue mucosa showed similar basal and predominantly parabasal distribution of cyclin D1-stained epithelial nuclei (D). CENP-F had the same distribution as seen in the epithelium from the floor of the mouth (E). Arrowheads indicate the parabasal layer in A–E; slightly larger arrowheads point to mitotic cells in the parabasal layer of the floor of the mouth stained for CENP-F in a slightly hyperplastic normal-appearing epithelium adjacent to a leukoplakia (F). Immunohistochemistry of proliferative markers counterstained with hematoxylin: A–E, ×100; and F, ×200.

epithelia. For Mib-1 comparisons, we pooled the normal data from the three intraoral sites.

Expression of Mib-1, Cyclin D1, and CENP-F in Leukoplakia. Positive nuclear staining for all three markers was identified in both LGD and HGD lesions. In LGD, staining of all markers was restricted to the basal and suprabasal layers (Fig. 4, A, C, and D), whereas in HGD, positively stained cells were also observed in the superficial layer (Fig. 4, B, E, and F).

The basal layer Mib-1 LI was significantly higher in both LGD and HGD than that of normal epithelia (approximately an 8-fold increase; Fig. 5; Table 1). The LI of the parabasal layer remained largely unchanged in LGD, whereas the LIs of the parabasal and superficial layers were higher in HGD than they were in LGD (approximately a 4.5-fold increase in the superficial layer).

In LGD, cyclin D1 showed similar changes, although the LI of the basal layer of LGD did not increase as markedly as the LI of Mib-1 (Fig. 5). Nevertheless, the basal layer LIs of both types of leukoplakia (HGD and LGD) were significantly different from the LI of normal epithelia (Table 1). The LIs of the HGD superficial layers were higher and statistically different from those of both LGD and normal epithelia (Table 1). The
Proliferative Markers in Oral Precancer

Basal Layer and Parabasal Layer

Fig. 2. Different LIs of the three proliferative markers in tongue epithelia derived from autopsies and biopsies. Differences were also seen in the other two sites (data not shown). Mean and SE are plotted.

superficial layer LI of HGD showed a 9-fold increase over the LOD cyclin D1 LI (Table 1; Figs. 4C and 5).

CENP-F changes in the leukoplakic epithelia were very similar to those described for cyclin D1 (Figs. 4, D–F, and 5). There was a remarkable increase in the basal layer LI in LGD and HGD (approximately a 7-fold increase over that of normal epithelia; Fig. 5), whereas the parabasal LI changed less drastically but also showed an increase in both types of leukoplakia. The superficial layer LI was 0 in normal epithelia and in LGD, whereas it was moderately high in the HGDs.

Basal layer Mib-1 (P < 0.001), superficial layer Mib-1 (P < 0.001), and superficial layer CENP-F (P < 0.001) were identified in the discriminant analysis as being jointly useful for distinguishing among the epithelial types. Using data from these three characteristics, 100.0% of normal epithelia and 88% of HGD were correctly categorized.

Discussion

The proliferative characteristics of normal oral epithelia and leukoplakias have been studied previously using mitotic counts and pulse labeling with tritiated thymidine or bromodeoxyuridine (3, 8, 32–35). Most of these reports have not specified the site of the oral mucosa studied or have focused on buccal mucosa. Although many studies have considered the deepest three epithelial layers as a single progenitor compartment (3, 36), others have divided this compartment into the basal and parabasal layers (34, 35). In addition, a recent report describes the LI of normal oral mucosa using immunohistochemical staining of PCNA (30). A comparison of our data (based on the quantitative analysis of Mib-1, cyclin D1, and CENP-F) with the other methods of proliferation evaluation is not easy. Nevertheless, some similarities are obvious. Several reports agree with our observations that the proliferative activity of normal oral epithelia occurs mostly in the parabasal layer and not in the basal epithelial cells (3, 34, 35). Kotelnikov et al. (35) describe LIs of 1.6 in the basal layer and 31.6 in the parabasal layer after iododeoxyuridine and bromodeoxyuridine pulse injection. Maidhof and Hornstein (34) describe a [3H]thymidine LI of 6.2 in the basal cells and 19.8 in the parabasal layer of the normal buccal epithelium. These figures are lower than our estimation of Mib-1 LI but compare favorably with our cyclin D1 LI (i.e., 2 in the basal layer and 23.6 in the parabasal layer of the buccal mucosa, respectively). These data most probably reflect the fact that Mib-1 detects all actively cycling cells, whereas cyclin D1 is expressed predominantly in keratinocytes in the G1-S phases. CENP-F figures are much lower, due to the fact that the G2-M phases of the cell cycle are shorter. Shin et al. (30) reported LIs of 6 and 7 for the basal and parabasal layer, respectively, using PCNA as a proliferation marker. The relatively low LI of the parabasal layer could be due to the sensitivity of the different proliferation markers used (Mib-1 is usually considered more reliable than PCNA) to the different sites studied, or to the fact that those samples were from six nonsmokers, whereas ours were mostly from smokers. Nevertheless, a comparison of the 3 markers in our samples from 15 smokers with the 5 samples from nonsmokers showed no statistically significant differences.

It is also noteworthy that the LIs of the autopsy-derived oral epithelia were consistently different from the LIs of the epithelia obtained by biopsy. Because this was probably an artifact, due to the longer and less strict conditions of prefixation and fixation of the tissues, we decided to use only the biopsy material for our analysis.
Fig. 4. Immunohistochemical detection of the three proliferative markers in dysplastic leukoplakia. Mib-1 immunostaining was seen in both the basal and parabasal layers of LGDs (A), whereas HGDs showed Mib-1 immunostaining in the basal, parabasal, and superficial layers (B). Similarly, cyclin D1 was seen in all three layers of HGD (C). CENP-F was localized in both the basal and parabasal layers of LGD (D). In addition, this marker was also localized in the superficial layer of HGD (E). In these lesions, CENP-F could be seen very clearly in the cytoplasm of mitotic cells (arrowheads) and in the nuclei of cells presumably in the G2 phase of the cell cycle (F). Immunohistochemistry of proliferative markers counterstained with hematoxylin: A–E, ×100; and F, ×200.

Regional differences in epithelial morphology and cell kinetics have been described in different sites of the oral mucosa (30, 37–39). In the present study, statistically significant differences among the tongue epithelium and the other two normal epithelia were found in the cyclin D1 and CENP-F LIs. Thus, in our comparative study with abnormal epithelia, we used tongue epithelium data for these two markers, whereas pooled Mib-1 data from the three sites was used for that marker because there were no statistically significant differences among the three intraoral epithelial sites. The use of tongue epithelium as a control for cyclin D1 and CENP-F was favored, because most dysplastic leukoplakias were from the tongue epithelium, and because it had the highest LI of the three normal oral epithelia; in the worst of cases, this would only underestimate the relative LI increase in dysplastic epithelia.

The increased proliferative capacity of leukoplakia has been recognized by several laboratories (8, 35, 36, 40, 41). Recently, similar conclusions have been reached using immunohistochemically detectable proliferation markers such as PCNA and Ki-67 (9, 12, 13, 42). In a detailed quantitative study of PCNA expression in head and neck lesions, Shin et al. (30) showed a gradual increase in the PCNA LI in all three layers, reaching a maximum in dysplastic epithelia. Similar conclusions could be reached from our material, in which HGD showed an increased LI of the three markers. This was especially true in the superficial layer, in which HGD showed a
marked increase with respect to normal epithelium and LGD. Another interesting change was the increase in the basal layer LI of all leukoplakias. This abrupt change from an almost negligible number of positive cells in the basal layer of the normal epithelia to a considerable number of positive cells in this layer in leukoplakias has been previously reported using autoradiographic techniques with tritiated thymidine (8) and immunohistochemistry with PCNA (9, 13). Thus, comparison of our data with those of the literature showed that Mib-1 is a reliable marker able to differentiate normal epithelium from a considerable number of positive cells in the basal layer of the normal tongue epithelium was used as a reference. Mean and SE are plotted.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>LIs of normal mucosa and dysplastic leukoplakiasa</th>
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</thead>
<tbody>
<tr>
<td>A.</td>
<td></td>
</tr>
<tr>
<td>Mib-1</td>
<td></td>
</tr>
<tr>
<td>NM (57)</td>
<td>LGD (30)                  HGD (17)</td>
</tr>
<tr>
<td>Basal layer</td>
<td>5.35 ± 2.50</td>
</tr>
<tr>
<td>Parabasal layer</td>
<td>51.28 ± 22.96</td>
</tr>
<tr>
<td>Superficial layer</td>
<td>0.00</td>
</tr>
<tr>
<td>B.</td>
<td></td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
</tr>
<tr>
<td>NMb</td>
<td>LGD                      HGD</td>
</tr>
<tr>
<td>Basal layer</td>
<td>2.87 ± 2.94</td>
</tr>
<tr>
<td>Parabasal layer</td>
<td>34.70± 13.86</td>
</tr>
<tr>
<td>Superficial layer</td>
<td>0.00</td>
</tr>
<tr>
<td>C.</td>
<td></td>
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<tr>
<td>CENP-F</td>
<td></td>
</tr>
<tr>
<td>NMb</td>
<td>LGD                      HGD</td>
</tr>
<tr>
<td>Basal layer</td>
<td>1.80 ± 1.57</td>
</tr>
<tr>
<td>Parabasal layer</td>
<td>8.91 ± 4.68</td>
</tr>
<tr>
<td>Superficial layer</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Fig. 5. LIs of dysplastic leukoplakias. LGDs and HGDs were compared with normal oral epithelia (NM). The data from the three intraoral sites were pooled to comparatively evaluate Mib-1 LIs. For cyclin D1 and CENP-F LIs, the normal tongue epithelium was used as a reference. Mean and SE are plotted.

...difications and invasive tumors is not only associated with a larger fraction of G1-S-phase cells but is due to molecular abnormalities that include, among other changes, gene amplification and rearrangements. In the present study, we have not analyzed these possible changes, but it is quite feasible that a significant proportion of leukoplakias contain molecular abnormalities that result in cyclin D1 overexpression.

The number of cases with cyclin D1 expression in precursor lesions of the larynx was seen to increase in direct proportion to the degree of atypia (20). The cyclin D1 staining in these precursor lesions was seen in the suprabasal cell layers (20). Although these results are mainly descriptive and are based on other intraoral sites, they coincide with our quantitative data of increased expression in the parabasal and superficial layers of oral leukoplakias. In our hands, this marker seems useful and sensitive, and it is able to differentiate both normal epithelium from LGD and LGD from HGD.

CENP-F is a new cell cycle-specific proliferation marker that to our knowledge has not been used in the evaluation of head and neck lesions. CENP-F is a kinetochore protein of 367 kDa that was originally identified by an autoimmune serum. It accumulates in nuclei of G2 cells and is degraded rapidly after the completion of mitosis (29). Recently, CENP-F has been used as a promising marker of cell proliferation in selected cases of breast cancer, lymphomas, and leukemias (21). Our data also indicate that this marker is quite sensitive and is able to detect subtle changes in proliferative activity in oral epithelium and precursor lesions.

In conclusion, the three markers used in this study to detect proliferative changes in oral precursor lesions are useful. All three layers investigated showed differences. Nevertheless, the basal and superficial layers showed the most clear differences between normal and abnormal tissues. Because of the higher number of cells and the relatively high proliferative activity in dysplastic leukoplakias, the superficial layer and the basal layer seem to be the most adequate tissue components in which to investigate the possible modulation of cell proliferation in precursor lesions treated with chemopreventive agents.

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References


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