Morphometric and immunohistochemical investigation of oral epithelial dysplasia and squamous cell carcinoma

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Abstract: Morphometric assessment including epithelial indices which express morphological features of the epithelium, mitotic index, mean nuclear area, mean form factor of the nucleus and cellular infiltration in the stroma was performed in 14 cases with oral non-dysplastic epithelium and 66 cases with dysplastic epithelium. The results from morphometry showed a close relationship to the histological severity of dysplasia determined by the histological criteria of Bánocz. Immunohistochemical localization of carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA) and epidermal growth factor (EGF) were investigated in 14 cases of non-dysplastic epithelium, 66 cases of dysplastic epithelium and 16 cases of squamous cell carcinoma, and compared with the morphometric results. Positive rates of CEA and EMA reaction in epithelial dysplasia increased with the advance of the dysplastic grade. Those of EGF reaction decreased with the advance of the dysplastic grade. It is suggested that morphometry and immunohistochemistry are useful in confirmation of the histological severity of oral epithelial dysplasia.

Introduction

There is no doubt that early diagnosis and treatment is important in squamous cell carcinoma of the oral cavity. A possible key to the early detection of carcinoma may lie in the precancerous mucosal changes that often precede oral cancer. Clinically, these lesions usually take the form of leukoplakia and/or erythroplakia. Microscopically, epithelial cellular changes such as atypia or dysplasia may indicate a precancerous potential. Pindborg et al. reported that in a 7-year followup study of 107 cases of oral epithelial dysplastic precancerous lesions in Indian villagers, 6.6 per cent were found to develop into carcinomas. Similar risk rates for malignant transformation have been reported. It is generally believed with a few exceptions that mild degrees of epithelial dysplasia do not indicate any great danger for the patient. Moderate dysplasia calls for a more cautious approach, while severe dysplasia indicates that there is a very considerable risk of cancer development. Therefore, it is extremely important to grade the histological severity in oral epithelial dysplasia. However, in attempts at an objective interpretation of the microscopic features of oral leukoplakia, the descriptions are still somewhat subjective and susceptible to observer variations. Pindborg et al. pointed out the lack of objectivity in evaluating precancerous lesions of the oral cavity. Furthermore, histological diagnosis is qualitative since the intensity of the features are not entirely reflected.
Smith and Pindborg advocated the atypia scoring technique, which is qualitative and semiquantitative. Morphometry is an objective and reproducible method for morphological observations. It has been able to quantify the intensity of histological features. Recently this method has been applied to pathological diagnosis and research. Quantitative histopathology has also been of great value.

On the other hand, immunohistochemistry is one of the advanced methods for histological evaluation of oral precancerous lesions and carcinomas. This study was undertaken in order to:

1. compare the conventional histopathological results with morphometric ones in oral epithelial dysplasia.
2. identify and compare immunohistochemical localization of carcinoembryonic antigen (CEA), epithelial membrane antigen (EMA), and epidermal growth factor (EGF) in non-dysplastic epithelium, epithelial dysplasia, and squamous cell carcinoma.
3. compare the morphometric results with immunohistochemical ones.
4. determine whether any of the immunohistochemical reagents could be investigated as a marker for histological screening.

Materials and methods

Materials

Biopsy specimens from 96 patients who suffered from oral leukoplakia or oral squamous cell carcinoma were fixed in 10% formalin and embedded in paraffin for examination. Clinical diagnosis of leukoplakia was carried out according to the criteria of WHO. Histological diagnosis of squamous cell carcinoma was determined by the WHO standard.

Preparation of specimens

Serial 4 μm sections were obtained from each specimen, one of which was routinely processed with hematoxylin and eosin staining to confirm histological diagnosis and morphometric assessment. The other adjacent sections were used for the immunohistochemical investigation.

Histological diagnosis

The 96 specimens were classified as non-dysplastic epithelium (14 cases), dysplastic epithelium (66 cases), and squamous cell carcinoma (16 cases). Dysplastic epithelium was graded as mild when two of the listed histological changes as shown in Table 1 were present, moderate when three to four changes were noted, and severe when five or more of the changes were present based on the criteria of Bánóczy.

Non-dysplastic epithelium had no observable histological deviations from normal epithelium.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Histological changes in epithelial dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Irregular epithelial stratification</td>
</tr>
<tr>
<td>2.</td>
<td>Hyperplasia of basal layer</td>
</tr>
<tr>
<td>3.</td>
<td>Drop-shaped rete pegs</td>
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<tr>
<td>4.</td>
<td>Increased number of mitotic figures</td>
</tr>
<tr>
<td>5.</td>
<td>Loss of polarity of basal cells</td>
</tr>
<tr>
<td>6.</td>
<td>Increased nuclear-cytoplasmic ratio</td>
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<tr>
<td>7.</td>
<td>Nuclear polymorphism</td>
</tr>
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<td>8.</td>
<td>Nuclear hyperchromatism</td>
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<tr>
<td>9.</td>
<td>Enlarged nucleoli</td>
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<tr>
<td>10.</td>
<td>Keratinization of single cells or cell groups</td>
</tr>
<tr>
<td>11.</td>
<td>in the prickle layer</td>
</tr>
<tr>
<td></td>
<td>Loss of intercellular adherence</td>
</tr>
</tbody>
</table>

(Bánóczy, J., 1976)

Morphometric assessment

The following parameters were determined in H. E. sections.
1. BML/SL = basement membrane length/surface length
2. AREA/SL = epithelial area/surface length
3. MI: mitotic index = number of mitoses/1 mm² surface area
4. CI: cellular infiltration in the stroma
5. MNA: mean nuclear area
6. MFF: mean form factors of the nucleus

Precautions were taken so that the selected specimens were not cut tangentially. Basement membrane length (BML), surface length (SL) and epithelial area (AREA) were measured using a Leitz A. S. M. semiautomated image analyzer on tracings described from H. E. sections through a drawing attachment (Nikon, Japan) set on a Nikon Optiphot microscope at ×40 magnification.
Correction of magnification was carried out using an objective micrometer (Nikon, Japan). Fig. 1 shows a schematic diagram of a histological section. SL is the length between A and B. BML is the length of the curved line between C and D. AREA is the area of figure ABDC. A, B, C and D are situated in the margin of the section. Line AC and BD is set up to be straight. BML/SL and AREA/SL express morphological features of proliferative epithelium, which are called epithelial indices for convenience. The higher the BML/SL value increases, the longer the rete pegs proliferate. The AREA/SL value is related to the entire thickness of the epithelium. The number of mitoses per 1 square millimeter of epithelial surface was determined using the system described by Karring and Löe.14

![Fig. 1](image)

Fig. 1 A schematic diagram of a histological section. SL is the length between A and B. BML is the length of the curved line between C and D. AREA is the area of figure ABDC (diagonal shaded area). Point A, B, C and D is in the margin of the section. The grade of cellular infiltration in the stroma was rated on a scale of 0 to 3 (0=no or less cellular infiltration, 1=mild, 2=moderate, 3=marked) beneath the epithelium based on the criteria of Anneroth.15

Mean nuclear area was measured as follows: \[ 4 \cdot \pi \cdot (\text{nuclear area}) / (\text{nuclear perimeter})^2 \], which is 1 for circle and 0 for irregular structures, indicative of roundness of the nuclei and the regularity of their profiles.11 Statistical evaluation was performed with the Wilcoxon’s rank sum test (W-test), Chi-square contingency test with Yates correction ($\chi^2$-test), and Fisher’s exact probability test (F-test) in cases in which one of the values was smaller than 4. Arithmetic means and standard deviations are quoted in the results for ease of presentation and comparison but were not used in the statistical analysis.

**Immunohistochemical assessment**

**Antisera**

The antiserum against CEA was purchased from Serotec Inc., England, which was absorbed with human spleen powder to remove non-specific cross-reacting antigen (NCA). The antiserum against EMA was purchased from Lipshaw Co. Ltd., U. S. A., and anti-EGF from Wakunaga Pharmacology Co. Ltd., Japan (Table 2). The antiserum against EGF was developed by injecting highly purified EGF prepared from a genetic-engineered E. coli host into New Zealand white rabbits. Anti-EGF rabbit serum was purified by EGF affinity chromatography. This antibody has been shown to be EGF-specific
and to have no cross reactivity with other peptides, including mouse EGF, fibroblast growth factor, platelet derived growth factor, endothelial cell growth supplement, secretin, glucagon, cholecystokinin, and endorphin\textsuperscript{16}.

**Immunohistochemical Procedures**

The sections were deparaffinized in xylene and rehydrated with water. After rinsing in phosphate-buffered saline (PBS), 3% hydrogen peroxide was used for 5 min. to block endogenous peroxidase activity. A protein blocking agent was incubated for 20 min. to block non-specific antibody staining. The sections were then incubated with the primary polyclonal antibodies (Table 2). Antibodies against CEA and EGF were diluted with PBS at pH 7.4. Secondary biotinylated antibody, and peroxidase reagent were added to the sections. At all stages, the specimens were incubated for 20-30 min. After each step, sections were extensively washed in PBS. Finally, peroxidase activity was revealed by incubating the slides with 3-amino-9-ethylcarbazol (AEC) and hydrogen peroxide for 5-10 min, then counterstaining with hematoxylin in some sections. Immunoperoxidase staining kit for the Avidin Biotin Affinity method were purchased from Lipshaw Co. Ltd., U. S. A. The slides were mounted in a glycerol-gelatin (Sigma, Co. Ltd., U. S. A.).

**Controls**

1) normal sheep/goat/rabbit serum was used as the first layer.

2) PBS was used as the first layer instead of the primary antibody, if necessary.

In addition for EGF,

3) The sections were incubated in the solution containing anti-EGF mixed with excess EGF at 4°C for 24 hours.

### Results

1. **Histological diagnosis**

Sixty-six cases of oral epithelial dysplasia were graded according to the criteria of Báñóczy\textsuperscript{8}. The distribution of mild, moderate and severe dysplasia in the sample is shown in Table 3. There were 18 cases (27.3%) with scores of 2 (mild dysplasia), 32 cases (48.5%) with scores of 3-4 (moderate dysplasia) and 16 cases (24.2%) with scores of more than 5 (severe dysplasia) (Table 3).

| Table 3 Distribution of cases by grade of epithelial dysplasia |
|-----------------|--------|--------|
| score           | No. of case | %     |
| 2 (mild)        | 18      | 27.3  |
| 3-4 (moderate)  | 32      | 48.5  |
| 5- (severe)     | 16      | 24.2  |
| non-dysplastic  | 66      | 100   |
| squamous cell carcinoma | 14 |        |
| epithelium     |         |       |

The percentage distribution of the histological changes in this series almost approximated those presented by Báñóczy\textsuperscript{8}. The age distribution of 66 patients with epithelial dysplasia is shown in Fig. 2. Peak frequency was in the 7th decade, followed by the 6th and 5th decade. The M : F ratio was 1 : 1.44.

The tongue was the site most frequently involved (34.8 %), followed by the gingiva (33.3 %), cheek (19.7 %), floor of mouth (9.1 %), and palate (3.1 %). The degree of dysplasia in relation to site is shown in Table 4. Three cases (13.1 %) involving the tongue, 6 cases (27.3 %) in the gingiva, and 5 cases (38.5 %) in the cheek were severely dysplastic.

| Table 4 Distribution of cases by site of epithelial dysplasia |
|-----------------|--------|--------|
| Site            | No. of case | %     |
| Tongue          | 23      | 34.8  |
| Gingiva         | 18      | 27.3  |
| Cheek           | 13      | 19.7  |
| Floor of mouth  | 6       | 9.1   |
| Palate          | 2       | 3.1   |

### Table 2 Antisera

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Animal</th>
<th>Laboratory</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carcinoembryonic antigen (CEA)</td>
<td>sheep</td>
<td>Serotec, England</td>
<td>1 : 200</td>
</tr>
<tr>
<td>(absorbed with human spleen powder)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial membrane antigen (EMA)</td>
<td>goat</td>
<td>Lipshaw, U.S.A.</td>
<td></td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>rabbit</td>
<td>Wakunaga, Japan</td>
<td>1 : 60</td>
</tr>
</tbody>
</table>
Peak frequency in epithelial dysplasia was between 1.50 and 2.00. The $M \pm S. D.$ was 2.48 ±1.07 in epithelial dysplasia, and 1.42±0.63 in non-dysplastic epithelium. The difference between epithelial dysplasia and non-dysplastic epithelium was significant ($p<0.01$, W-test). The BML/SL values in severe dysplasia were significantly higher than those in mild and moderate dysplasia ($p<0.01$, W-test).

2) AREA/SL

The results of AREA/SL in non-dysplastic epithelium and epithelial dysplasia are shown in Fig. 3 and Fig. 6. Peak frequency in epithelial dysplasia was between 0.4 and 0.6. The $M \pm S. D.$ was 0.52±0.25 in epithelial dysplasia and 0.28±0.14 in non-dysplastic epithelium. The AREA/SL values in epithelial dysplasia were significantly higher than those in non-dysplastic epithelium ($p<0.01$, W-test). The AREA/SL values in severe dysplasia were significantly higher than those in mild dysplasia ($p<0.01$, W-test) and moderate dysplasia ($p<0.05$, W-test). Correlation coefficient ($r$) between the BML/SL and AREA/SL values was 0.60. Positive linear correlation was obtained between AREA/SL and BML/SL values.

3) MI

The results of MI in non-dysplastic epithelium and epithelial dysplasia are shown in Table 5. The smallest MI value was 0 in

<table>
<thead>
<tr>
<th>Site</th>
<th>Degree of dysplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mild</td>
</tr>
<tr>
<td>tongue</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(30.4%)</td>
</tr>
<tr>
<td>gingiva</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>(31.8%)</td>
</tr>
<tr>
<td>cheek</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(7.7%)</td>
</tr>
<tr>
<td>floor of mouth</td>
<td>2</td>
</tr>
<tr>
<td>hard palate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>
Fig. 3 Epithelial indices (BML/SL, AREA/SL). BML/SL and AREA/SL values increase with the advance of dysplastic grade. Significant differences are observed as the following:

BML/SL: p < 0.01; a vs c, a vs d, b vs d, c vs d, p < 0.05; a vs b
AREA/SL: p < 0.01; e vs g, e vs h, f vs h, g vs h, p < 0.05; e vs f

ND: non-dysplastic epithelium
mild: mild epithelial dysplasia
moderate: moderate epithelial dysplasia
severe: severe epithelial dysplasia

non-dysplastic epithelium, mild and moderate dysplasia. The largest value was 2152 in severe dysplasia. The M ± S. D. was 429 ± 527 in epithelial dysplasia, and 106 ± 120 in non-dysplastic epithelium. The S. D. was large in both groups, which indicates a scattering of each value in the same group. The difference between epithelial dysplasia and non-dysplastic epithelium was significant (p < 0.05, W-test).

The largest MI value in moderate dysplasia was 1962. The mean value was 296. However, the MI values in moderate dysplasia were not significantly higher than those in non-dysplastic epithelium and mild dysplasia. Peak frequency in severe dysplasia was between 500 and 600. The MI values in severe dysplasia were significantly higher than those in mild and moderate dysplasia (p < 0.01, W-test).

4) CI (Cellular infiltration in the stroma)

Cellular infiltration in the stroma was lymphocytic dominant in 69% of the cases with epithelial dysplasia. Lymphocyte infiltration equivalent to that of plasma cells was observed in 12% of the cases. Dominant plasma cell infiltration was observed in 16% of the cases. The results of CI in non-dysplastic and epithelial dysplasia are shown in Table 6 and Fig. 7. The minimum CI value was 0 in non-dysplastic epithelium, mild and moderate dysplasia and 1 in severe dysplasia. The maximum value was 2 in non-dysplasia and mild dysplasia, and 3 in moderate and severe dysplasia. Peak frequency in non-dysplastic epithelium, mild and moderate dysplasia was at 1 and that in severe dysplasia was at 3. The difference between epithelial dysplasia and non-dysplastic epithelium was
Fig. 5-A  CEA reaction in epithelial dysplasia (ABC with hematoxylin ×50).
CEA distributions were seen in the superficial layer and circumferentially in the parakeratinized layers (arrow).

Fig. 5-B  CEA reaction in squamous cell carcinoma (ABC ×50).
Strongly positive stainings were frequently seen in the zone of keratotic differentiation.

Fig. 5-C  EMA reaction in dysplastic epithelium (ABC ×25).
Intra-epithelial keratinization in the spinous cell layer showed strongly positive staining. Half or all of the cytoplasmic membrane of the parakeratinocytes and spinous cells were weakly positive.

Fig. 5-D  EMA reaction in squamous cell carcinoma (ABC ×40).
Strongly positive staining in the cytoplasmic membrane and weakly positive staining in the cytoplasm of the tumor cells are observed.
5) MNA (Mean Nuclear Area)

The MNA value was obtained from every 40 nuclei in the basal cell layer, lower spinous cell layer and upper spinous cell layer of the cases with epithelial dysplasia. The minimum MNA value was $21.9\pm8.0\ \mu m^2$ (M±S. D.), and the maximum value was $89.3\pm16.8\ \mu m^2$ (M±S. D.). Peak frequency in the basal cell layer and lower spinous cell layer was between 40 and $50\ \mu m^2$, and between $50$ and $60\ \mu m^2$ in the upper spinous cell layer. Most of MNA values in the spinous cell layers were higher than those in the basal cell layers. The MNA values obtained from the three layers of epithelium in the severely dysplastic cases were significantly higher than those from mildly dysplastic cases ($p<0.05$, W-test).

6) MFF (Mean Form Factor of the nucleus)

The MFF values were obtained from the three layers of epithelium, similar to the MNA values. The MFF values ranged from $0.74\pm0.13$ to $0.90\pm0.06$ (M±S. D.) in the basal cell layer. Peak frequency in the three layers were between 0.85 and 0.90. The MFF values in lower and upper spinous cell layers were higher than those in basal cell layer, which indicated the shape of the nuclei in spinous cell layers were more nearly circular compared with those in the basal cell layer. There was no significant difference in MFF values (W-test).

3. Immunohistochemical results

1) CEA

#1 non-dysplastic epithelium

All specimens showed no CEA localization in the epithelium (Fig. 4).

#2 epithelial dysplasia

CEA distribution was found in the cytoplasmic membrane of the cell in the superficial parakeratinized layers and spinous cell layers (Fig. 5-A). The basal cell layers were negative. The rate of positive reactions showed slight increase with the advance of the dysplastic grade (Fig. 4).

#3 squamous cell carcinoma

CEA reaction in squamous cell carcinoma was found in the epithelial pearls and cytoplasmic membrane of tumor cells. CEA ex-
pressed cells were frequently seen in the zone of keratotic differentiation (Fig. 5-B). There was little reaction of CEA in poorly-differentiated carcinoma. The staining intensity increased with the amount of keratinization. The epithelium adjacent to infiltrating squamous cell carcinomas also showed weakly positive reactions. The overall positive rate of CEA in squamous cell carcinoma was 87.5% (14/16). Immunoreactivities in epithelial dysplasia and squamous cell carcinoma were absent in the sections incubated in normal sheep serum instead of anti-CEA.

2) EMA

#1 non-dysplastic epithelium
CEA reaction was negative in the non-dysplastic epithelium (Fig. 4).

#2 epithelial dysplasia
EMA localization was found from the superficial layers to the lower spinous layers. The basal cell layers were negative in most cases. However, in one case, EMA reaction was confined to the basal cell layer. EMA localization was found in the cytoplasmic membrane. A strongly positive reaction was found in relation to the intra-epithelial keratinization of the epithelium (Fig. 5-C). Staining was limited to half of the cytoplasmic membrane of the spinous cells in some specimens. The rate of positive reaction increased with the advance of the dysplastic grade (Fig. 4).

#3 squamous cell carcinoma
EMA localization was found in the zone of keratotic differentiation. Well-differentiated carcinomas were usually more extensively positive than poorly-differentiated lesions. EMA staining was most marked in well and moderately-differentiated cells with ample cytoplasm and well developed prickles. The positive cells almost always stained around their full circumference (Fig. 5-D). The epi-

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**Table 5** MI value in non-dysplastic epithelium and epithelial dysplasia

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Range</th>
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<tbody>
<tr>
<td>non-dysplastic epithelium</td>
<td>106</td>
<td>0-371</td>
</tr>
<tr>
<td>mild dysplasia</td>
<td>231</td>
<td>0-904</td>
</tr>
<tr>
<td>moderate dysplasia</td>
<td>296</td>
<td>0-1962</td>
</tr>
<tr>
<td>severe dysplasia</td>
<td>917</td>
<td>132-2152</td>
</tr>
</tbody>
</table>

**Table 6** CI value in non-dysplastic epithelium and epithelial dysplasia

<table>
<thead>
<tr>
<th></th>
<th>Mean ± S.D.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-dysplastic epithelium</td>
<td>0.86±0.77</td>
<td>0-2</td>
</tr>
<tr>
<td>mild dysplasia</td>
<td>0.78±0.65</td>
<td>0-2</td>
</tr>
<tr>
<td>moderate dysplasia</td>
<td>1.44±0.67</td>
<td>0-3</td>
</tr>
<tr>
<td>severe dysplasia</td>
<td>2.44±0.73</td>
<td>1-3</td>
</tr>
</tbody>
</table>

**Fig. 6** Relationship between EGF reaction and epithelial indices.

Solid oval shows positive EGF reaction in the case, and open oval shows negative EGF reaction. Positive rates of EGF reaction in the group with AREA/SL<0.48 are higher than those in the group with AREA/SL≥0.48 (p<0.05, Chi² test).
Fig. 7 Relationship between EMA reaction and CI value.
Solid oval: EMA positive case
Open oval: EMA negative case
CI: Cellular infiltration in the stroma
DG: dysplastic grade
Positive rates of EMA reactions in the CI=2, 3 group are significantly higher than those in the CI=0, 1 group (p<0.05, Chi² test).

3) EGF

#1 non-dysplastic epithelium
EGF localization was found in the superficial layers and upper spinous cell layers. Positive staining was found in the cytoplasm (Fig. 4).

#2 epithelial dysplasia
Positive reactions were found in the cytoplasm of spinous cells and keratinized cells (Fig. 5-E). Parakeratinized cells also showed intense positive reaction. Staining intensity decreased from surface to deeper layers. The rate of positive reactions and staining intensities decreased with the advance of the dysplastic grade (Fig. 4).

#3 squamous cell carcinoma
Weakly positive stainings were found in the cytoplasm of tumor cells which formed keratinized pearls in 6 of 16 squamous cell carcinoma specimens (37.5%, Fig. 4, 5-F). Immunohistochemical reactivity was negative in the sections incubated with normal rabbit serum and the anti-serum absorbed with excess EGF instead of the anti-EGF.

4. Relationship between morphometric and immunohistochemical results

Each morphometric value was divided into two groups to examine the relationship between morphometric and immunohistochemical results in epithelial dysplasia. In dividing into two groups, the median of each morphometric value was used as a border. As to the CI values, those were divided into the two groups; CI=0, 1 (none or mild cellular infiltration) and CI=2, 3 (moderate or marked).

1) Relationship between epithelial indices and immunohistochemical results
The BML/SL values were divided into the two groups; BML/SL≥2.33 and BML/SL<2.33 (Fig. 6). There was no significant difference in the positive rates of CEA, EMA, and EGF reaction between the two groups.

The AREA/SL values were divided into two groups; AREA/SL≥0.48 and AREA/SL<0.48 (Fig. 6). Positive rates of EGF reaction in lower AREA/SL group were significantly higher than those in higher AREA/SL group (p<0.05, Chi² test). There was no significant difference in the positive rates of CEA and EMA reaction between the two groups.

2) Relationship between MI values and immunohistochemical results

The MI values were divided into two groups; MI≥263 and MI<263. There was no significant difference in the positive rates of CEA, EMA and EGF reaction between the two groups.

3) Relationship between CI values and immunohistochemical results

The CI values were divided into two groups; CI=0,1 (none-less) and CI=2,3 (moderate-marked) (Fig. 7). Positive rates of EMA reactions in the CI=2,3 group were significantly higher than those in the CI=0,1 group (p<0.05, Chi² test). There was no significant difference in positive rates of CEA and EGF reaction between the two groups.

4) Relationship between MNA values and immunohistochemical results

The MNA values were also divided into two groups using the median of each of the MNA values (Fig. 8). In three layers, positive rates of EGF reactions in lower MNA group were significantly higher than those in higher MNA group (p<0.05, Chi² test). There was no significant difference in positive rates of CEA and EMA reactions between the two groups.

5) Relationship between MFF values and immunohistochemical results

The MFF values were divided into two groups using median of each of the MFF values in three layers (Fig. 8). There was no significant difference in the positive rates of CEA, EMA and EGF reactions between the two groups.

Discussion

I. Morphometry

Epithelial indices

In this study, epithelial indices including BML/SL and AREA/SL values were measured. The former expresses elongation of the basement membrane to surface length, and the latter expresses thickness of epithelium. Epithelial indices seemed to correspond with the histological changes of "drop-shaped rete pegs and irregular epithelial stratification". These histological changes were subjective assessments that the intensity of these changes may not be easily understood by other observers. Significant differences in BML/SL and AREA/SL were observed between non-dysplastic epithelium and epithelial dysplasia, also between mild, moderate and severe dysplasia. This suggested that these values increased with the advance of the dysplastic grade and were diagnostic with respect
Mitotic index

In assessing the mitotic activity, the following indices have been used:

1) the number of mitoses per 1 square millimeter of the epithelial surface\(^{17}\),
2) the number of mitoses per 1000 cells\(^{18}\),
3) the number of mitoses per 10 millimeter of basement membrane\(^{19}\).

The results from this study were obtained with the first index, which resulted in significant differences between mild, moderate and severe dysplasia. This fact indicated that quantitative evaluation of the increased number of mitotic figures was important in assessing oral epithelial dysplasia and that MI values might bring a more exact determination of the histologic severity of dysplasia.

The value of BML/SL ratio, one of the epithelial indices, showed extreme variations with the advance of dysplastic grade. Surface length showed smaller variations in this study. These findings suggested that the number of mitotic figures per unit surface, rather than per unit basement membrane is the relevant measure in dysplastic epithelium. The rate per 1 millimeter surface length could also be a reliable index because the difference between rate per unit surface area and that per unit surface length is the same with or without multiplication of the section thickness\(^{14}\).

Cellular infiltration in the stroma

Cellular infiltration is generally considered a defensive reflex. This reaction is considered to be one of the factors in the tumor-host relationship of squamous cell carcinoma\(^{15,20}\). There were few data available on the stromal reaction in epithelial dysplasia. Lehner\(^{21}\) has shown that the round cell infiltrate beneath oral epithelium increases progressively from benign keratoses through varying degrees of atypia to carcinoma.

Lönning and Burkhardt\(^{22}\) studied histologic changes of the tongue in 150 post-mortem cases and showed that all examined cases exhibited inflammatory cell infiltration of varying intensity in the subepithelial stroma, particularly immune competent cells. The reaction in the stroma was more marked in the higher grades of dysplasia.

The results of the present study showed similar findings to those of Lönning and Burkhardt\(^{22}\). Namely, cellular infiltration increased with the advance of the dysplastic grade, and that in severe dysplasia was significantly higher than in mild and moderate dysplasia. Significant differences were also observed between non-dysplastic epithelium and dysplastic epithelium. Recent studies with immunohistochemical detection to phenotypes of mononuclear inflammatory cells have clarified the role of the immunocompetent cells in oral epithelial dysplasia\(^{1,23,24}\).

Mean nuclear area and mean form factor

The nucleus is essential for the life of the cell, since it contains the genetic material of the cell and has a direct influence on the metabolic activities of the cytoplasm. The volume of the nucleus is related to its DNA content and, therefore, the number of chromosomes, and there is evidence that the volume increases with synthetic activities of the cell\(^{25}\). Nuclear variability in size, shape and structure represents the basic system of “nuclear” grading\(^{26}\). By means of morphometric assessment of histological and nuclear grades, high reproducibility has been introduced, according to van Bogaert et al.\(^{27}\) and Baak et al.\(^{28}\).

Nuclear features such as perimeter, area, longest and shortest axis, axis ratio, shape factor, and size may add to the discriminant power of the mitotic activity index and cellularity index which seem to be the best histological discriminators\(^{29,29}\).

Histological features in epithelial dysplasia included several nuclear changes, such as an increased nuclear–cytoplasmic ratio, increased number of mitotic figures, enlarged and increased nucleoli, and nuclear hyperchromatism\(^{3,8}\). In a morphometric study of dysplasia and carcinoma of the cervix, Hillemanns et al.\(^{30}\) related nuclear volume and cytoplasmic-nuclear ratio to the various stages of dysplasia and carcinoma. They found that there was a steady rise in nuclear volume, and a fall in the cytoplasmic-nuclear ratio through the stages of dysplasia to carcinoma in situ. When invasion had started, the nuclear volume started to fall again.

Kinoshita et al.\(^{31}\) reported on nuclear
DNA content and nuclear area in oral squamous cell carcinoma and precancerous stages. They stated that enlargement of the nuclear area was observed from normal through dysplastic epithelium to squamous cell carcinoma with the increase of nuclear DNA, and that several cases of epithelial dysplasia showed increased nuclear DNA and nuclear area similar to cells of squamous cell carcinoma. They speculated that these cases seemed to have malignant potential which could not be predicted from conventional histological assessment.

In this study, mean nuclear area (MNA) values obtained from the three layers of the epithelium in severe dysplasia was significantly higher than those in mild dysplasia. These findings suggested that increased MNA values may be an important factor in determination of the grade of epithelial dysplasia. Prediction of nuclear roundness and profile of their regularity showed no significant difference, even if the grade of dysplasia increased with the enlargement of the nuclei. MFF seems not to be diagnostic in the studies of oral epithelial dysplasia with light microscopy.

II. Immunohistochemistry

A development made possible by improvements in methods such as immunoperoxidase and avidin-biotin-complex-technique allows for application of such tests to paraffin sections of fixed tissues. Immunohistochemistry is a useful tool as well as morphometry in diagnostic tumor pathology. For example, the usefulness is observed in distinguishing carcinoma from sarcoma, distinguishing reactive lesions from carcinoma, and detecting micrometastases in histological sections. Various antigenic constituents, such as epithelial surface antigens, intracellular components/products, constituents of the basement membrane zone, and stromal changes have been examined by immunohistochemistry in oral precancerous lesions and carcinoma. In this study, immunohistochemical localization of CEA, EMA and EGF were investigated. These immunohistochemical reagents all survive in routine processed paraffin-embedded sections.

Carcinoembryonic antigen

Carcinoembryonic antigen (CEA) is a glycoprotein of 180000-200000 daltons that was initially found in extracts of colonic adenocarcinoma by Gold and Freedman. As the name implies, this material can be found in both fetal endoderm and transformed carcinoma arising in organs derived from this endoderm. Subsequently, it has been shown to be present in many types of tumors as well as normal tissue, plasma and other body fluids, with the differences being quantitative rather than qualitative.

Distribution of CEA has been demonstrated in squamous cell carcinomas of the parotid gland. However, CEA was found to contain non-specific cross-reacting antigen. Kramer reported no distribution of CEA in oral carcinomas using the antibodies absorbed with normal human spleen. Toto showed the presence of CEA in oral squamous cell carcinoma using an immunofluorescent technique. Yanagawa et al. demonstrated immunoperoxidase positive staining of CEA in 40% of oral squamous cell carcinoma, and 77.8% of squamous cell carcinomas of the maxillary sinus using the antibody absorbed with human spleen powder. They also showed that plasma CEA levels in the advanced stage patients were significantly higher than those in early stage patients. They concluded that in patients having high levels of pretherapy plasma CEA and the occurrence of CEA in the primary tumors, plasma CEA levels can be useful to monitor the effectiveness of treatment. In the present study, non-dysplastic epithelium showed no distribution of CEA. In contrast, CEA was observed in 87.5% of the squamous cell carcinoma sections. The positive rate of CEA reaction gradually increased with the advance of the dysplastic grade. These findings suggested that the expression of CEA might have a relation to dysplastic proliferation of epithelium.

Epithelial membrane antigen

Epithelial membrane antigen (EMA) is a large molecular weight glycoconjugate which can be identified in formalin-fixed paraffin-embedded sections by standard immunohistochemical techniques using antisera to human milk fat globule membranes. In normal
tissues, it is present on the luminal membrane of transitional epithelium. This might suggest a role of EMA in the secretory process. EMA is widely distributed in, and is confined to the epithelium and mesothelium and is expressed by many tumors derived from them. Its usefulness has been confirmed in differential diagnosis of the tumor, presuming the tumor is of epithelial or mesothelial origin. Sloane et al. investigated the distribution of EMA in human squamous epithelium using immunocytochemical techniques. The antigen was not demonstrable in normal adult tissues although it is present on the surface membrane of normal fetal epidermis before keratinization takes place. It may be expressed strongly in the adult, however, in infiltrative and in situ squamous cell carcinomas as well as in a variety of non-neoplastic states. In the present study, the positive rate of EMA reaction was 0% in non-dysplastic epithelium, and gradually increased with the advance of the dysplastic grade. The rate in severe dysplasia was higher than that in mild dysplasia. All of the squamous cell carcinomas showed EMA localization varying with the intensity.

One of the purposes of this study was to determine whether any of immunohistochemical reagents could be investigated as a marker for histologic screening of epithelial dysplasia. An ideal marker would be expressed on the surface of neoplastic epithelium and be absent from non-neoplastic tissue. EMA fulfills these requirements most closely. Adjacent dysplastic epithelium to squamous cell carcinoma, which might have a similar biological feature to that of squamous cell carcinoma, also showed EMA positivity. In addition, EMA distributions were frequently observed in the severely dysplastic epithelium. These findings suggest that EMA is a useful marker for histological screening of epithelial dysplasia.

Bamford et al. indicated that in inflammatory disorders, EMA concentrated in those cells in or near zones of inflammatory cell infiltration, and that EMA distribution was often confined to the basal layer. In the present study, EMA expression was often observed in the entire thickness of the epithelium. This seemed to be related to the dysplastic changes rather than inflammation.

Epidermal growth factor

Epidermal growth factor is a low molecular weight polypeptide isolated by Cohen from the extracts of mouse submaxillary gland. It induced precocious eyelid opening and incisor eruption due to a direct stimulation of epidermal growth and keratinization, when injected into newborn animals. An homologous polypeptide, human epidermal growth polypeptide, human epidermal growth factor was isolated from human urine. Urogastrone, a gastric antisecretory hormone isolated from human urine, is probably identical to human EGF. EGF stimulates proliferation of various cultured cells including epidermal keratinocytes and tumor cells. EGF has been shown to be secreted in human urine, saliva and milk. It was found that there was no EGF localization in the tongue by the use of radioimmunoassay. Kasselberg et al. reported immunohistochemical localization of EGF in the following tissues: submandibular gland, stomach, duodenum, adult lactating breast, adult eccrine sweat gland and kidney. This incidence was considered to be due to the higher sensitivity in the immunoperoxidase method than that in radioimmunoassay.

In this study, immunohistochemical distributions of EGF were found in the sections of non-dysplastic epithelium, epithelial dysplasia, and squamous cell carcinoma. There was no immunohistochemical distribution in the sections incubated in the antiserum absorbed with excess EGF and normal rabbit serum instead of primary antiserum. According to Kasselberg et al., EGF appeared to be secreted onto epithelium which is subjected to trauma, i.e., in sweat onto the skin and in saliva into the mouth. This supports immunohistochemical distributions of EGF in the oral squamous epithelium.
The expression of EGF and staining intensity gradually decreased from mild to severe dysplasia and from superficial to deeper layers. Positive rates of EGF reaction decreased with advance of dysplastic grade. Positive rates of EGF reaction in the lower MNA group, which is considered to approximate the nuclear area of normal oral squamous epithelium in paraffin-embedded sections, were significantly higher than those in the higher MNA group. Furthermore, positive rates of EGF reaction in the lower AREA/SL group were significantly higher than those in the higher AREA/SL group. These incidences suggest that EGF may be a useful marker to recognize mild epithelial dysplasia.

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