Loss of basement membrane components laminin and type IV collagen parallels the progression of oral epithelial neoplasia

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Aims: To determine the immunohistochemical localization of basement membrane components laminin and type IV collagen in premalignant and malignant lesions of the oral epithelium.

Methods and results: Formalin-fixed tissue sections of 12 epithelial hyperplasias with no dysplasia and 30 dysplasias, clinically diagnosed as leukoplakia and/or erythroplakia, as well as 50 invasive squamous cell carcinomas, were stained with mouse monoclonal antibodies to human laminin and type IV collagen. Statistical analysis showed that there was a linear trend for discontinuous distribution of laminin from epithelial hyperplasia to epithelial dysplasia and invasive squamous cell carcinoma (P < 0.001). Laminin staining showed a linear trend for discontinuity with increasing grade of dysplasia (P < 0.05) and was more frequently discontinuous in areas of deep tumour invasion than in central or superficial areas (P < 0.05). Brush-shaped thickening and reduplication of the basement membrane were also identified.

Conclusions: Alterations in the distribution of laminin and type IV collagen in oral premalignant and malignant lesions indicate that the loss of continuity of the subepithelial basement membrane parallels the progression of the neoplastic transformation process in oral epithelium.

Keywords: basement membrane, carcinoma, immunohistochemistry, laminin, leukoplakia, oral, squamous cell, type IV collagen

Introduction
Basement membranes are complex extracellular matrices of specialized structure and function, present in all vertebrate and most invertebrate animals. They separate epithelial, endothelial and mesothelial cells from the underlying connective tissue, or surround vascular pericytes and muscle, adipose and Schwann cells. Unique components of basement membranes include type IV collagen, laminin, perlecan, nidogen/entactin, type VII collagen and some minor or site-specific molecules. Basement membranes participate in tissue development, growth, maintenance, repair and regeneration. They provide structural support, form selective barriers and are involved in critical cellular functions such as movement, attachment, proliferation and differentiation.

Neoplastic invasion and metastasis are characterized by the ability of tumour cells to cross tissue compartment boundaries. The subepithelial basement membrane plays an important role in the complex interactions of this process, as it is the first obstacle to be traversed by the neoplastic cells. In general, the subepithelial basement membrane is lost in most invasive carcinomas, whereas in benign lesions and in situ carcinomas its continuity is retained. The ability of malignant neoplasms to destroy the basement membrane has been related to their invasive potential, and utilized as an aid in the early diagnosis and prediction of the biological behaviour of various tumors.

Oral squamous cell carcinomas account for 5% of all cancers occurring in the human body and comprise
more than 90% of mouth malignancies. A portion of carcinomas are preceded for several months or years by premalignant or precancerous lesions, usually leukoplakia or erythroplakia, whose histopathological appearance varies from epithelial hyperplasia with no dysplasia, to various grades of epithelial dysplasia or in situ carcinoma. The malignant transformation rate of precancerous lesions ranges from 4% to 35%, and is closely related to the degree of epithelial dysplasia present.

The study of basement membrane in premalignant and malignant oral lesions may enhance our understanding of the biological and clinical behaviour of individual lesions. Most reports are focused on squamous cell carcinoma, where laminin, type IV collagen, heparan sulfate proteoglycan and type VII collagen distribution have been related to clinical and histopathological parameters of known prognostic value.

The aim of the present study was to investigate the immunohistochemical localization of laminin and type IV collagen in the subepithelial basement membrane zone of oral premalignant lesions and invasive squamous cell carcinomas.

**Materials and methods**

**TISSUE SPECIMENS**

The material investigated was collected retrospectively from the files of the Division of Oral Pathology, Faculty of Dentistry, University of Athens. It included 12 cases of epithelial hyperplasia with no dysplasia (keratosis and/or acanthosis), 30 cases of epithelial dysplasia, and 50 cases of invasive squamous cell carcinoma. Clinically, epithelial hyperplasias and dysplasias had been diagnosed as leukoplakia and/or erythroplakia. Sites of occurrence of the lesions are listed in Table 1. The specimens were obtained as diagnostic incisional biopsies or definite surgical resections from previously untreated patients and routinely fixed in 10% formalin and embedded in paraffin. Ten samples of normal buccal, lingual and labial mucosa were utilized as controls.

Histopathological examination was performed on 5 μm thick haematoxylin and eosin stained sections. The grade of epithelial dysplasia and the degree of differentiation of squamous cell carcinomas were determined according to standard criteria. Carcinomas were also classified according to the multifactorial malignancy grading system of Byrne et al., which evaluates the degrees of keratinization and nuclear pleomorphism, the mode of invasion and the stromal plasma-lymphocytic inflammatory infiltration at the deeper, invasive front of the tumour.

**Table 1. Sites of occurrence of epithelial hyperplasia with no dysplasia, epithelial dysplasia and squamous cell carcinoma**

<table>
<thead>
<tr>
<th>Histological diagnosis</th>
<th>Site of occurrence</th>
<th>Tongue</th>
<th>Lips</th>
<th>Buccal mucosa</th>
<th>Gingivae</th>
<th>Floor of mouth</th>
<th>Palate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial hyperplasia</td>
<td></td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>11</td>
</tr>
<tr>
<td>Epithelial dysplasia</td>
<td></td>
<td>13</td>
<td>2</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>–</td>
<td>27</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td></td>
<td>23</td>
<td>3</td>
<td>9</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>47</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>41</td>
<td>7</td>
<td>22</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>85</td>
</tr>
</tbody>
</table>

**IMMUNOCYTOCHEMISTRY**

Five-μm thick step sections were mounted on glass slides covered with Vectabond bonding agent (Vector Laboratories Inc., Burlingame, CA; SP-1800) and stained with a standard three-step avidin–biotin–complex method (Vectastain® Elite ABC kit, PK-6102). Deparaffinized and rehydrated tissue sections were immersed in 1.5% H₂O₂ for 10 min in a dark place, in order to block endogenous peroxidase activity. After washing in tris-buffered saline (TBS, pH 7.6) the sections were digested with 2 mg/ml pepsin (Sigma Chemical Co., St. Louis, MO; P7012) in 0.1 M HCl for 30 min, at 37°C for laminin and 22°C for type IV collagen, for optimal antigen retrieval. Further washing was followed by saturation of the sections with nonimmune serum diluted in TBS supplemented with 1% bovine serum albumin (BSA). Incubation with mouse monoclonal antibodies to human laminin (clone LAM89; Biomakor bm, Kryiat Weizmann, Rehovot, Israel, 6072, lot no. L9Q208) and type IV collagen purified from human kidney (clone CIV22; Dako, Glostrup, Denmark, M785, lot no. 039) diluted at

1:2000 and 1:50, respectively, in TBS–1% BSA, was performed overnight at 4–5°C. The specificity of the antibodies has been previously characterized.21,22 The sections were washed in TBS, incubated with the biotinylated secondary antibody, diluted in TBS–1% BSA, washed again in TBS and treated with the ABC complex, diluted in TBS–1% BSA. Antibody binding was visualized with 60 mg/100 ml 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, MO; D-5637) and 0.003% H2O2. Sections were counterstained with Harris' haematoxylin, dehydrated, cleared and mounted. For the definition of negative controls the primary antibodies were omitted.

The staining pattern of laminin and type IV collagen was subjectively evaluated at a standard magnification (×100) along the basement membrane zone of normal mucosa and premalignant lesions, and around the tumour nests of squamous cell carcinomas. Staining was characterized as continuous when a brown reaction product was seen along the epithelial–mesenchymal border, or discontinuous when the reaction was fragmentary or absent. Areas with disruption of the epithelium due to heavy stromal inflammatory infiltration, were excluded from the study.

Because of the known continuous distribution of laminin and collagen IV around blood vessels, submucosal salivary glands, nerves and fascicles of skeletal muscle, these structures served as intrinsic positive controls. For statistical analysis the Fisher’s exact test in two-way contingency tables and the chi-squared test for trend were calculated at a 5% significance level.

Results

A regular and continuous brown line beneath the basal cell layer of the epithelium, consistent with the basement membrane zone, was present in all samples of normal mucosa stained for laminin and type IV collagen (Figure 1). The distribution of laminin and type IV collagen in normal oral mucosa, epithelial hyperplasia with no dysplasia, epithelial dysplasia and squamous cell carcinoma is summarized in Table 2. Cases excluded showed repetitious negative immunostaining of intrinsic controls or proteolytic destruction. The staining pattern of both antibodies was more frequently continuous in epithelial hyperplasia with no dysplasia (Figure 2) and epithelial dysplasia, than in carcinoma. Overall, there was a highly significant linear trend for discontinuous distribution of laminin from epithelial hyperplasia with no dysplasia to epithelial dysplasia and invasive squamous cell carcinoma (P < 0.001).

Sixteen cases of epithelial dysplasia were graded as mild, seven cases as moderate and seven cases as severe or in situ carcinoma (Table 3). Statistical analysis showed that there was a significant linear trend for discontinuous laminin distribution with the increasing grade of dysplasia (P < 0.05) (Figure 3).

Fifteen cases of squamous cell carcinoma were graded...
as well differentiated, 28 cases as moderately differentiated and seven cases as poorly differentiated (Table 4). Well and moderately differentiated carcinomas exhibited continuous staining (Figure 4) more frequently than poorly differentiated tumours (Figure 5), but the trend was not statistically significant. Malignancy grading\(^\text{20}\) could be applied on 41 carcinomas (Table 5). Nine cases totalled 4–8 grades, 22 cases 9–12

**Table 3.** The pattern of laminin and type IV collagen immunohistochemistry in epithelial dysplasia in relation to the grade of dysplasia

<table>
<thead>
<tr>
<th>Grade of dysplasia</th>
<th>Laminin</th>
<th>Collagen IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Continuous</td>
</tr>
<tr>
<td>Mild</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Moderate</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Severe (carcinoma in situ)</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>24</td>
</tr>
</tbody>
</table>

Laminin: \(P = 0.0221\) according to \(\chi^2\) for trend; collagen type IV: \(P = 0.2345\) according to \(\chi^2\) for trend.

as well differentiated, 28 cases as moderately differentiated and seven cases as poorly differentiated (Table 4). Well and moderately differentiated carcinomas exhibited continuous staining (Figure 4) more frequently than poorly differentiated tumours (Figure 5), but the trend was not statistically significant. Malignancy grading\(^\text{20}\) could be applied on 41 carcinomas (Table 5). Nine cases totalled 4–8 grades, 22 cases 9–12

**Table 4.** The pattern of laminin and type IV collagen immunohistochemistry in squamous cell carcinoma in relation to the degree of differentiation

<table>
<thead>
<tr>
<th>Degree of differentiation</th>
<th>Laminin</th>
<th>Collagen IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Continuous</td>
</tr>
<tr>
<td>Good</td>
<td>14</td>
<td>7</td>
</tr>
<tr>
<td>Moderate</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>Poor</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>18</td>
</tr>
</tbody>
</table>

Laminin: \(P = 0.1118\) according to \(\chi^2\) for trend; collagen type IV: \(P = 0.2377\) according to \(\chi^2\) for trend.
grades and 10 cases 13–16 grades. There was no statistically significant correlation between the staining pattern and the grade of malignancy. Staining for laminin was more frequently discontinuous in areas of deep tumour invasion than in central or superficial areas, the difference being statistically significant ($P < 0.05$, Fisher’s exact test) (Table 6).

The thickness of normal and lesional basement membrane zone presented wide regional variations. Three cases of epithelial hyperplasia with no dysplasia, the superficial epithelium in six cases of carcinoma and the nondysplastic tumour-adjacent epithelium of one carcinoma, exhibited brush-shaped thickenings of the basement membrane zone (Figure 6), which reacted with both antibodies or only with CIV 22 (four carcinomas). A case of epithelial hyperplasia with no dysplasia and the mildly dysplastic and hyperplastic tumour-adjacent epithelium of one carcinoma showed linear stromal depositions of basement membrane markers that were in continuity with the lesional epithelium (Figure 7). Step sectioning revealed that those configurations reacted both for laminin and type IV collagen. Focal cytoplasmic immunoreactivity for either laminin or type IV collagen was present in two carcinomas.

### Discussion

Oral premalignant lesions and squamous cell carcinoma represent consecutive steps of the multistep tumorigenesis process and are a unique model for the *in vivo* study of basement membrane involvement in epithelial carcinogenesis. The immunohistochemical localization of basement membrane markers may also provide additional diagnostic and prognostic information to that of the morphological features assessed by routine histopathology.

Meyer et al. found that the distribution of laminin and fibronectin in normal oral mucosa, leukoplakia and squamous cell carcinoma was not significantly different, but they did not specify the histopathological diagnoses of the leukoplakias studied. Firth and Reade reported that the distribution of laminin and type IV collagen was continuous in epithelial hyperplasia, while dysplastic lesions showed small focal breaks whose number was increased in more severe dysplasias. As these lesions were not the main focus of their investigation, the number of cases included was too small for evaluation. Kannan et al. noticed a gradual increase in the frequency of discontinuity of laminin and type IV collagen from normal through hyperplastic, dysplastic

<table>
<thead>
<tr>
<th>Tissue compartment</th>
<th>Laminin</th>
<th>Collagen IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Continuous</td>
<td>Discontinuous</td>
</tr>
<tr>
<td>Superficial-central</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Invasive</td>
<td>15</td>
<td>26</td>
</tr>
</tbody>
</table>

Laminin: $P = 0.0146$ according to Fisher’s exact test; collagen type IV: $P = 0.5077$ according to Fisher’s exact test.
and malignant epithelium, a trend statistically documented for laminin in the present series. It should be noticed that all epithelial hyperplasias with no dysplasia studied, were not related by the contributing clinicians to any certain aetiological factor, and it is more likely that they do represent neoplastic lesions.

The progressive alteration of basement membrane in epithelial transformation may be a sign of the appearance of clonal cell populations with an invasive phenotype. The trend for more frequent variation of the basement membrane in dysplasias of higher grade, statistically documented for laminin in the present study, is in agreement with this argument, as the risk for infiltrative growth increases in parallel to the severity of the dysplasia. A similar trend has been noticed in cervical intraepithelial neoplasias, dysplasias of the upper aerodigestive tract and chemically induced skin dysplasias. The variability of basement membrane staining in dysplasias of the same grade, as well as the presence of alterations in hyperplasias, does not contradict this view, as the clinicopathological features of oral premalignant lesions do not always reflect the biological potential of individual cases.

Laminin and type IV collagen could be detected in all cases of oral squamous cell carcinomas but tumour islands of well and moderately differentiated neoplasms exhibited a higher percentage of continuous basement membrane than poorly differentiated lesions. The relationship of basement membrane reaction to the differentiation of oral carcinomas has been previously noticed and statistically documented by Kumagai et al. No correlation was found between the distribution of laminin and type IV collagen and the grade of malignancy which has better predictive value and reproducibility than the degree of differentiation, and incorporates the pattern of cancer invasion whose correlation to the basement membrane staining pattern has been previously shown.

Figure 4. Continuous immunohistochemical distribution of laminin around the tumour nests of a well-differentiated squamous cell carcinoma (original magnification ×220).

Figure 5. Absence of collagen type IV immunoreactivity in the deep infiltrative margin of a poorly differentiated squamous cell carcinoma. Note positivity of small blood vessels (arrows) and muscle bundles (arrowheads) (original magnification ×130).

Figure 6. Laminin immunopositivity of brush-shaped thickenings of the basement membrane zone in an epithelial hyperplasia with no dysplasia (original magnification ×220).

Figure 7. Linear stromal depositions of collagen type IV in an epithelial hyperplasia with no dysplasia that are in continuity with the lesional epithelium (arrows) (original magnification ×130).
The more frequent loss of basement membrane component in the invasive front than the central or superficial areas of oral squamous cell carcinomas, statistically significant for laminin in the present series, has been previously described but not documented in carcinomas of the oral cavity,11,14,18,23 as well as carcinomas of the head and neck.13,30 Skin and stomach,12 and adenocarcinomas of the uterine cervix.13 Active proteolytic degradation by specific enzymes and decreased production or deficient assembly of basement membrane components by functionally altered neoplastic cells are crucial for the loss of continuity of basement membrane in cancer.79 Immunohistochemical studies have shown both increased expression of matrix degrading enzymes25,34 and phenotypical abnormalities35 at the tumour front of oral carcinomas. Thus, these defects may reflect an invasive, migrating, and proliferative phenotype.35 In fact, Harada et al.9 found that the staining pattern of laminin, type IV collagen and heparan sulphate proteoglycan at the invasive front of primary oral squamous cell carcinomas was similar to that in lymph node metastases and concluded that the cellular population of the deeper areas expresses the invasive and metastatic potential of the oral carcinomas. From a practical point of view, this finding indicates that the invasive front should be the field of study of basement membrane alterations in oral squamous cell carcinomas.

The variation of the thickness of basement membrane among premalignant and malignant oral lesions previously reported24 could not be confirmed in this study, as in most specimens the membrane was locally loosened or widened, preventing objective evaluation. Brush-shaped thickenings of laminin or collagen type IV Similar to those noticed in premalignant lesions and the noninvasive compartment of carcinomas in the present study have not been previously reported in oral lesions. The reaction of these structures with both antibodies indicates that they constitute projections of the basement membrane zone.

The linear, stromal depositions of basement membrane markers seen are similar to the reduplications reported by Gusterson et al.9,36 in solar keratosis, in situ carcinoma and squamous cell carcinoma of the skin. Reduplications have been noticed also in non-neoplastic skin lesions such as lichen planus and lupus erythematosus, and are thought to represent subepithelial basement membrane residues after regression of the lesion and shift in the position of the initial epithelial–stromal interface.9 Accordingly, reduplications were found to react with both membrane markers tested and were seen in a case of nondysplastic leukoplakia whose potential for regression is established. Areas reminiscent of reduplication were seen in a few squamous cell carcinomas of the present series but the origin of these depositions from adjacent, compressed stromal elements, such as muscle bundles or small vessels, could not be rejected.

The results of the present study confirm the alterations in the distribution of laminin and type IV collagen in premalignant and malignant lesions of the oral epithelium and indicate that the loss of continuity of the subepithelial basement membrane parallels the progression of the neoplastic transformation process. However, prospective studies are needed for the clarification of the biological significance of these changes.

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References


