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Distribution of Basal Lamina Type IV Collagen and Laminin in Normal Rat Tongue Mucosa and Experimental Oral Carcinoma: Ultrastructural Immunolocalisation and Immunogold Quantitation

De-Jun Jiang, David F. Wilson, Peter S. Smith, Angela M. Pierce and Ole W. Wiebkin

The relationship of basal lamina, a form of specialised extracellular matrix which separates epithelial cells and other cell types from adjacent stroma, to the behaviour of malignant neoplasms of epithelial origin is not well understood. However, it is widely acknowledged that the properties of local invasion and metastasis of carcinomas are linked to extracellular matrix (including basal lamina) changes. In the present study, the distribution of the major basal lamina components, type IV collagen and laminin, in normal rat tongue mucosa and experimentally induced oral carcinomas was investigated using post-embedding immunogold techniques and electron microscopy. The expression of these components was also quantitatively analysed using morphometry and immunocytochemistry. Results indicated that type IV collagen and laminin were confined to the lamina densa of normal oral epithelial basal lamina, and that both components were also detected in the lamina densa of basal lamina associated with carcinomas, and in the extracellular matrix of tumours. Furthermore, laminin was detected within stromal fibroblasts in normal tissues and experimental carcinomas. Quantitative analysis indicated that expression of laminin was significantly increased in carcinomas. In contrast, type IV collagen expression was significantly decreased. The quantitative changes observed in the two basal lamina constituents may be related to the process of tumour invasion, reflecting altered metabolic activities of tumour and stromal cells. These observations may be of use in understanding the architectural characteristics of oral mucosa basal lamina and in assessing the malignant potential of epithelial dysplasias or "premalignant" lesions.

Keywords: basal lamina, immunocytochemistry, laminin, morphometry, oral mucosa, quantitation, rat, squamous cell carcinoma, type IV collagen.
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INTRODUCTION

BASAL LAMINA is a form of specialised extracellular matrix which separates epithelial cells and other cell types from adjacent stroma. A number of basal lamina components have been identified, namely, type IV collagen, laminin, heparan sulphate proteoglycan, and entactin [1–4]. Among these, type IV collagen and laminin constitute the major components of basal laminae in various tissues. As described in previous studies [5–7], these basal lamina constituents play important roles in homoeostasis (that is, cell attachment, cell migration, cell differentiation and organisation of basal lamina) and in pathological situations such as tumour invasion and metastasis.

The role of basal lamina in relation to the behaviour of malignant neoplasms of epithelial origin is not well understood. However, it is widely acknowledged that the properties of local invasion and metastasis of carcinomas are linked to extracellular matrix (including basal lamina) changes [7, 8]. Morphological changes in basal lamina (for example, discontinuities, duplication, thicknening, intensive or absent staining) have been noted in various epithelial neoplasms including

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those of skin, cervix, breast, and oral mucosa, using electron microscopy and immunohistochemistry [9–14]. These alterations in tumour basal lamina have been related to tumour differentiation and behaviour [15] and there is a view that investigation of such changes will lead to a better understanding of the biology of cancers and improved means of diagnosis of these cancers [16], especially in the early stages. This is of particular relevance to oral squamous cell carcinoma which, although not prevalent, is associated with a high mortality rate that could be significantly reduced by earlier diagnosis, especially of premalignant lesions.

The distribution of basal lamina type IV collagen and laminin in both normal and neoplastic tissues has been studied by a number of investigators using optical immunohistochemistry [12, 14, 17–20]. These studies included examination of carcinomas from various sites and demonstrated intense staining of laminin and discontinuous staining of type IV collagen in tumour basal laminae [14, 19, 20]. Qualitative ultrastructural immunolocalisation of these components has also been described for such tissues as kidney, intestine and retinal capillaries [21, 22]. This research has contributed to our understanding of the structure–function relationships and the pathobiological process of neoplasia. However, there is little information available describing the localisation of type IV collagen and laminin in normal oral mucosa basal lamina and the ultrastructural distribution and quantitative changes in these basal lamina components in malignant epithelial neoplasms, including oral carcinoma.

In this study, the ultrastructural localisation of basal lamina type IV collagen and laminin in normal rat tongue mucosa and in 4-nitroquinoline-1-oxide (4NQO)-induced experimental oral carcinoma was investigated using post-embedding immunogold techniques and electron microscopy (immunocytochemistry). In addition, a quantitative evaluation of the expression of these components in both normal rat oral mucosa and experimental oral carcinomas was undertaken.

MATERIALS AND METHODS

A total of 30 female Wistar rats (average weight 180 g) were used and allocated to one of three groups:

(1) an untreated control group,
(2) a propylene glycol-treated control group, and
(3) a 4NQO-treated group.

Ten microlitres of 0.5% 4NQO in propylene glycol (vehicle) were applied three times a week for 24 weeks to the dorsal tongue of anaesthetised rates in the 4NQO-treated group. Rats in the propylene glycol-treated group only received propylene glycol, and the untreated group were sedated only.

After killing, suitably sized tissue blocks were obtained from the posterior dorsal two-thirds of tongues from group 1 and 2 animals, and from induced carcinomas in 4NQO-treated animals (for details of sampling protocols please refer to the Morphometry section). Specimens were immediately fixed by immersion in a solution of 0.25% glutaraldehyde-0.15% picric acid-5% sucrose in 0.1 mol/l phosphate buffer (PB) (pH 7.2, 365 mosm) for 1 h. After washing in 0.1M PB containing 50 mM NH_4Cl and 3% sucrose for 1 h, tissues were dehydrated through a series of graded ethanols (1 h in total) and infiltrated with pure, hard-grade L.R. White resin (L.R. White, London, U.K.) for 3 h at room temperature. Tissues were then embedded in this resin using the manufacturer's accelerator for 2–3 h at room temperature. Ultrathin sections (60–90 nm thick) were cut with a diamond knife and mounted on formvar-coated nickel grids.

In addition to tissue blocks processed for electron microscopy, paraffin-embedded normal tongue and carcinoma specimen blocks were also processed from the tongue of each animal. Sections cut from these blocks were stained with haematoxylin and eosin.

Immunocytochemistry

Grids with ultrathin sections were inverted onto a drop of the following solutions on paraffin film for staining:

(1) 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 5 min.
(2) Primary antibody at dilutions of 1:10 for rabbit anti-human laminin polyclonal antibody (EY Labs, California) or 1:5 for purified goat anti-human and bovine type IV collagen polyclonal antibody (Southern Biotechnology, Birmingham) in PBS containing 1% BSA and 1% Tween 20 for 3 h.
(3) Three PBS rinses for 5 min each.
(4) 1% BSA in PBS for 5 min.
(5) Gold-complex in PBS containing 1% BSA and 1% Tween 20 for 30 min at dilutions of 1:200 20 nm protein A-gold for laminin antibody or 1:20 20 nm rabbit antigoat IgG-gold (ICN Biochemicals, USA) for type IV collagen antibody.
(6) Three PBS rinses for 5 min each.
(7) Distilled water for 5 min.
(8) 2% aqueous uranyl acetate for 2 min.
(9) Reynold's lead citrate for 30 s.

Control incubations consisted of the following protocols:

(1) Primary antibody was replaced by PBS.
(2) Primary antibody was replaced by antibody-corresponding antigen absorption solution (anti-human laminin antibody was preabsorbed with an excess of 1 mg/ml purified laminin; anti-human and bovine type IV collagen antibody was preabsorbed with an excess of 2 mg/ml purified human and bovine type IV collagen).

Sections were viewed in a Jeol 100S electron microscope and examined for the presence of and distribution of gold particles indicating the respective antigens.

Morphometry

The posterior dorsal two thirds of the normal tongues and induced carcinomas were cut into 1–2 mm sagittal slices. Every second tissue slice was selected from each sample pool. One mm^3 (approximately) tissue blocks were cut from these tissue slices and 30 representive tissue blocks per tongue or per carcinoma were randomly selected for embedding. From these block pools, three blocks per tongue or per carcinoma were selected and subsequently one block per tongue or per carcinoma was randomly selected for sectioning. Twenty ultrathin sections (60–90 nm thick) per block were cut with a diamond knife and mounted on formvar-coated parallel line nickel grids. One ultrathin section per block was randomly selected for electron microscopic examination. Sections were viewed and five electronmicrographs were randomly selected using the same sampling patter (Fig. 1) for each section (one section per tongue or per carcinoma).

The sample size for this study was previously determined using the statistical "component of variance" model [23]. A

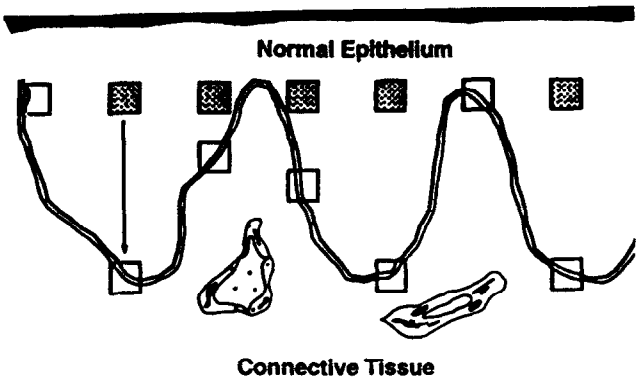

Fig. 1. Diagram showing the sampling pattern used for selecting electron micrographs in normal tissue. Areas of basal lamina were selected by taking equidistant photomicrographs in a horizontal plane across the section. Where a field (shaded) did not incorporate the basal lamina, a perpendicular was dropped until basal lamina was reached, and that field was used for photomicrography and counting. The horizontal distance between fields was 6 μm, measured using the micrometre drive of the microscope.

morphometric point-counting method [24] and MOP digitizer system (Carl Zeiss. Inc, Austria) were used to estimate basal lamina areas and length, respectively. The label density of basal lamina type IV collagen and laminin for lamina densa was represented by the number of gold particles per unit area and per unit length. For lamina lucida, only particles per unit area were measured as the ultrastructural definition of this structure did not allow accurate digitization. All data were subjected to analysis of variance using a computer software package (UCLA, ed Dixon, 1991).

RESULTS

Ultrastructural observation of normal specimens [10 group (1) normal animals and 10 group (2) propylene glycol control animals] showed a continuous basal lamina with distinct lamina lucida and lamina densa layers. Gold particles indicating laminin were distributed in the lamina densa of normal epithelial basal lamina (Fig. 2), and only a few gold particles were seen in the lamina lucida. None were detected in epithelial cells. In the connective tissue, blood vessel and nerve basal laminae and fibroblasts showed the presence of numerous gold particles (Fig. 3).

Immunogold labelling for type IV collagen was observed in the lamina densa of normal epithelial basal lamina (Fig. 4). Blood vessel basal laminae exhibited dense labelling, but epithelial cells and connective tissue cells were devoid of label. The distribution of mucosal epithelial basal lamina laminin and type IV collagen in normal tissues was consistent when individual animals were compared. There was no obvious difference in expression of basal lamina laminin and type IV collagen between group (1) normal tissues and group (2) propylene glycol-treated tissues.

Histological examination of paraffin sections from 4NQO treated animals showed that all 10 animals exhibited well-differentiated squamous cell carcinomas of the tongue. Electronmicroscopic examination of ultrathin sections revealed focal discontinuities and thickening of carcinoma basal laminae. An increased intensity of immunogold labelling for laminin was seen in the lamina densa of tumour basal lamina in all specimens (Fig. 5). In addition, gold particles were distributed in the tumour stroma, in some carcinoma cells and in stromal fibroblasts where labelling was observed in the cytoplasm and around nuclei. Immunogold labelling for laminin in neoplastic epithelial cells was not uniform in density or distribution and was only observed in a minority of cells. Labelling of carcinoma-associated stromal fibroblasts was seen in fewer cells compared with normal mucosal connective tissue.

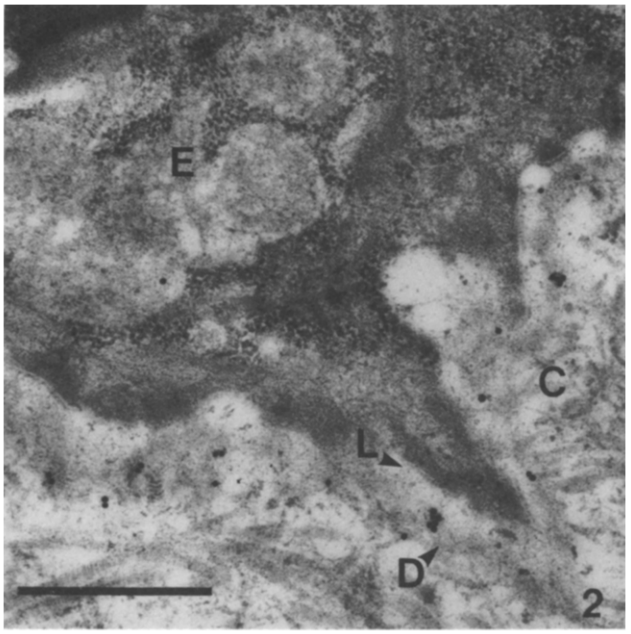

Fig. 2. Normal rat tongue mucosa. Electron-dense gold particles indicating laminin are evident in the lamina densa (D) of the basal lamina. L = lamina lucida, E = basal epithelial cell, C = connective tissue (bar = 0.5 μm).

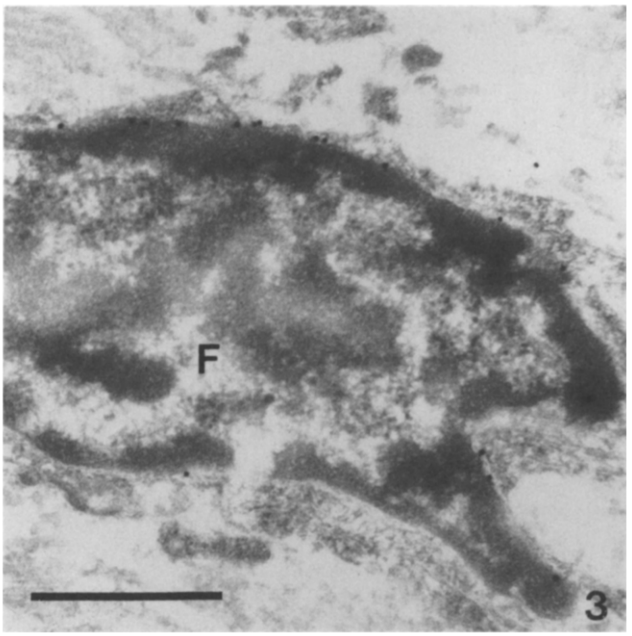

Fig. 3. Normal rat tongue mucosa. Perinuclear electron-dense gold particles indicating laminin are seen in stromal fibroblasts (F) (bar = 0.5 μm).

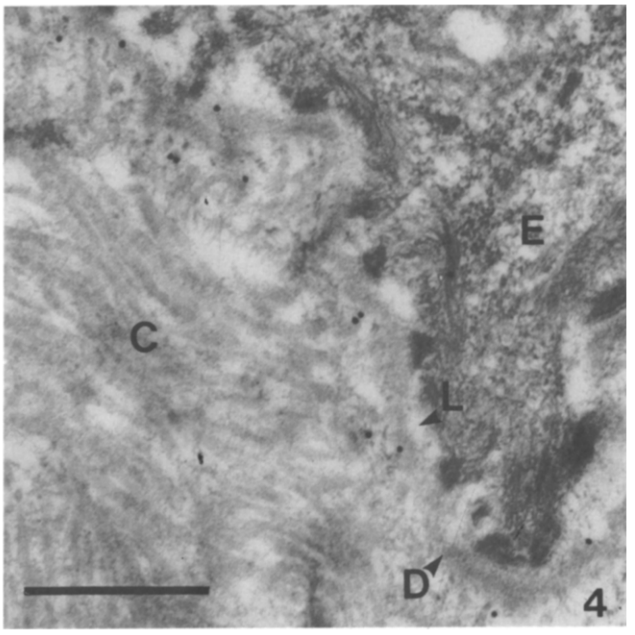

Fig. 4. Normal rat tongue mucosa. Electron-dense gold particles indicating type IV collagen are evident in the lamina densa (D) of the basal lamina. L = lamina lucida, E = basal epithelial cells, C = connective tissue (bar = 0.5 μm).

Immunogold labelling for epithelial basal lamina type IV collagen in carcinomas (Fig. 6) revealed variable expression, principally in the lamina densa at a level of intensity less than that observed for laminin in carcinomas. Occasional gold particles indicating type IV collagen were evident in the extracellular matrix of carcinoma stromal tissues.

All control sections showed negative immunogold labelling for type IV collagen and laminin.

Statistical analysis of quantitative data obtained using the methods described indicated that the label density of lamina densa laminin (number of gold particles per μm^2 area or per μm length) in the experimental oral carcinomas was significantly higher than that of the untreated group ($P < 0.01$) (Table 1). In contrast, the label density of lamina densa type IV collagen (number of gold particles per μm^2 area or per μm length) in the experimental oral carcinomas was significantly lower than that in the untreated group ($P < 0.01$) (Table 1). There were no significant differences ($P > 0.5$) in the label density of lamina densa type IV collagen and laminin (number of gold particles per μm^2 area or per μm length) between the untreated group and the propylene glycol-treated group (Table 1), and no significant differences ($P > 0.5$) were found in the label density of lamina lucida type IV collagen and laminin (number of particles per μm^2 area) among the three groups (that is, the untreated group, the propylene glycol-treated group and the carcinomas) (Table 2).

DISCUSSION

Previous studies have examined the distribution of basal lamina type IV collagen and laminin in a number of tissues: Foidart *et al.* [25] and Martinez-Hernandez and Chung [3] used immunoperoxidase techniques and electron microscopy to demonstrate the distribution of laminin in both the lamina densa and the lamina lucida of glomeruli, muscle and intestine, and in the lamina lucida of skin and oesophagus. Using immunogold labelling techniques and electron microscopy, laminin has been identified in the lamina densa and the lamina lucida of basal laminae in glomeruli, colon and enamel organ, and type IV collagen located in the lamina densa of glomeruli, colon and retinal vessels [21, 22, 26, 27]. Jungell [28], using an immunoperoxidase technique and electron microscopy, reported that type IV collagen and laminin were localised in both the lamina densa and lamina lucida of human oral mucosa. In the present study, we employed ultrastructural

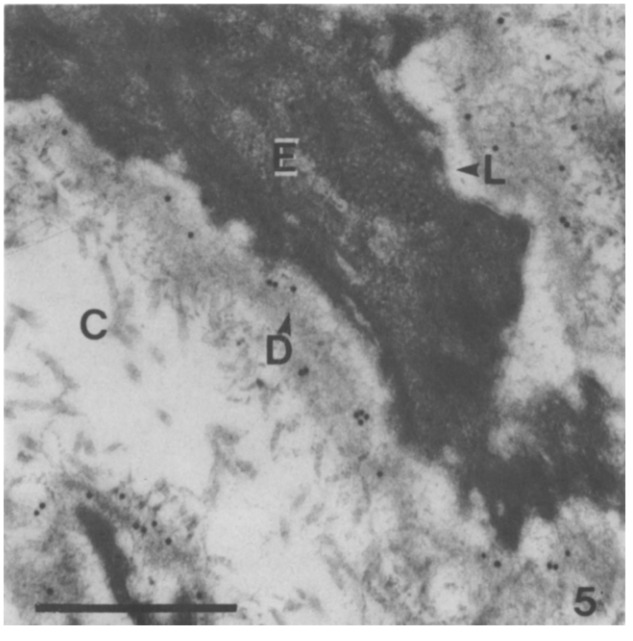

Fig. 5. Carcinoma. Electron-dense gold particles indicating laminin are evident in the lamina densa (D) of the basal lamina. L = lamina lucida, E = basal epithelial cells, C = connective tissue (bar = 0.5 μm).

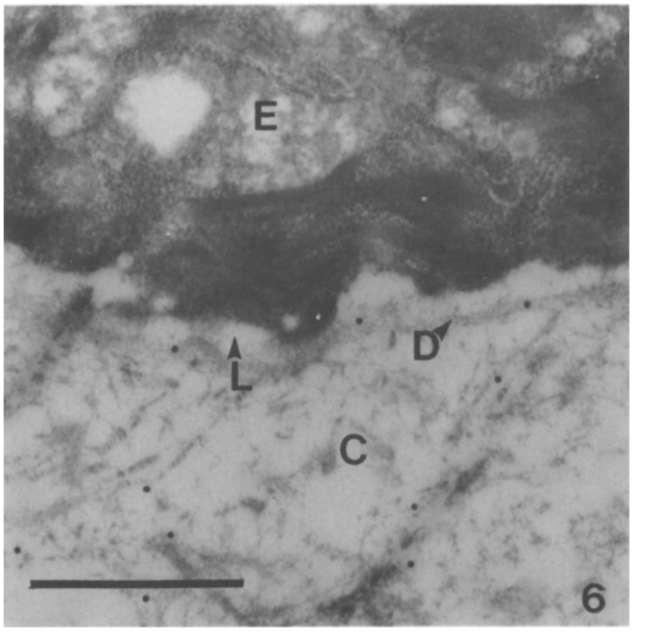

Fig. 6 Carcinoma. Electron-dense gold particles indicating type IV collagen are seen in the lamina densa (D) of the basal lamina and in the extracellular matrix. L = lamina lucida, E = basal epithelial cells, C = connective tissue (bar = 0.5 μm).

Table 1. Mean numbers of gold particles (indicating laminin or type IV collagen) per μm² or per μm of lamina densa from normal epithelial basal lamina, basal lamina from propylene glycol-treated mucosa and carcinoma basal lamina

Variable	Group		
	Untreated (Mean ± S.D.)	Propylene glycol (Mean ± S.D.)	Carcinoma (Mean ± S.D.)
Laminin (no./μm²)	30.25 ± 13.33	24.67 ± 11.08	66.66 ± 21.01*
Laminin (no./μm)	0.86 ± 0.39	0.70 ± 0.34	1.56 ± 0.60*
Type IV collagen (no./μm²)	26.81 ± 15.93	27.60 ± 12.31	14.01 ± 8.28*
Type IV collagen (no./μm)	1.28 ± 0.65	1.42 ± 0.73	0.73 ± 0.41*

*$P < 0.01$ between groups.

Table 2. Mean numbers of gold particles (indicating laminin and type IV collagen) per μm² of lamina lucida from normal epithelial basal lamina, basal lamina from propylene glycol-treated mucosa and carcinoma basal lamina

Variable	Group		
	Untreated (Mean ± S.D.)	Propylene glycol (Mean ± S.D.)	Carcinoma (Mean ± S.D.)
Laminin (no./μm²)	1.70 ± 1.09	1.17 ± 1.00	1.46 ± 1.11
Type IV collagen (no./μm²)	0.69 ± 0.87	0.63 ± 0.87	0.56 ± 0.86

Differences were not statistically significant ($P > 0.5$).

immunogold techniques to identify basal lamina type IV collagen and laminin in normal rat oral mucosa and in experimentally induced oral carcinomas and found that both basal lamina antigens were confined principally to the lamina densa in the basal lamina of both normal and carcinomatous epithelium.

Whether or not the apparent differences in location of basal lamina type IV collagen and laminin observed in the present study and in studies by others are due to intrinsic factors, such as the heterogeneity of molecular structure [27], or extrinsic factors (for example, the different sources of antibody) remains to be established. In immunological and biochemical studies, heterogeneity in the molecular structure of type IV collagen and laminin between species and tissues has been demonstrated [7, 29]. Moreover, several types of antibody specific for different epitopes of type IV collagen and laminin molecules have been raised, resulting in varying levels of detection of type IV collagen and laminin in basal laminae [30, 31]. In the present study, basal lamina type IV collagen and laminin were localised by the cross-reactivity of anti-human (and bovine) polyclonal antibody. It is possible that some antigenic epitopes of basal lamina type IV collagen and laminin in rat tissue are not recognised by these antibodies. However, notwithstanding this, our observations concerning the localisation of type IV collagen are consistent with the findings of the majority of studies by others [21, 22, 27], with the exception of Jungell's study where the diffuse immunoperoxidase labelling may have influenced the precision of localisation of type IV collagen [28].

In the present study, it was interesting to note that immunogold labelling for laminin was observed in stromal fibroblasts in both normal tissues and in experimental carcinomas. Laurie and colleagues [32] investigated the biosynthesis of laminin in the endodermal cells of rat parietal yolk sac using anti-laminin antibodies and immunoperoxidase techniques. Immunostaining was seen in cellular organelles (such as Golgi apparatus and rough endoplasmic reticulum) and it was suggested that the intracellular expression of laminin could be attributed to the presence of laminin precursors. Campbell and Terranova [7] reported that epithelial cells, endothelial cells, muscle cells and some types of fibroblasts were sources of laminin production, and Chung [33] described fibroblasts in prostatic cancer which produced extracellular matrix components including laminin. On the basis of these observations, it is suggested that the stromal fibroblasts which were seen to express labelling for laminin in the present study contain antigenic epitopes of laminin, and may be involved in the production of laminin.

Apart from stromal fibroblasts, some carcinoma cells exhibited intracytoplasmic staining. Previous studies have demonstrated that tumour cells synthesise large amounts of laminin [7] and Lee [15] suggested that the intracytoplasmic expression of laminin within carcinoma cells is related to the degree of differentiation (that is, cells in more poorly differentiated carcinomas express more laminin). In the present study, the experimentally-induced oral carcinomas were histologically well-differentiated lesions, and gold labelling detected within epithelial cells was relatively sparse and did not occur in all cells.

Morphometric methods have been widely used in various fields of biological science [24], including the quantitative evaluation of tissue structure and tissue antigens [21, 24, 34, 35]. In morphometric studies, accurate sampling is critical for obtaining an unbiased estimate [24, 36]. In the present study, all sampling procedures complied with the principle of random selection of samples. Previous studies have used a variety of sampling patterns for tissues such as kidney and oral mucosa [36–38]. In Schroeder's investigations, basal lamina regions at the base of epithelial ridges and above connective tissue papillae were randomly selected in order to estimate the

thickness of basal lamina in human oral mucosa [38]. In the present study, we employed a method which included the base of epithelial ridges, above the connective tissue papillae and the area between both of these regions. This pattern ensured random selection of both normal and neoplastic basal laminae samples.

In the quantitative evaluation of tissue antigen expression in sections, the label density of antigen is represented by the number of gold particles per area or per length in the given tissue compartment [21, 34]. In the present study, two methods were employed for evaluating basal lamina type IV collagen and laminin, namely point counting and digitising. It was found that the point-counting method was more accurate for estimating basal lamina area while the digitiser system was better suited for measuring basal lamina length.

The matter of random sampling aside, the determination of sample size is also considered to be critical for obtaining an unbiased estimate in a quantitative study [24, 37]. For the present study, final sample size was determined in a pilot study by analysing the variation at each level of sampling with the statistical "variance of component" model [23]. The pilot study confirmed that the number of basal lamina fields used in the present study was appropriate.

In previous investigations, descriptions of the changes in type IV collagen and laminin in neoplasms have focused on morphological staining patterns at light and electronmicroscopic levels [12, 14, 15, 17–20, 39, 40]. The quantitative ultrastructural data obtained in the present study provide evidence of differences in the amount of laminin and type IV collagen in the basal lamina of normal rat oral epithelium and carcinomas deriving from this tissue. The increased expression of laminin in tumour basal lamina possibly represents evidence of increased production of this substance in tumour tissue and may partly account for the increased quantity (thickness) of basal lamina observed in a number of tumour studies [13, 41]. Our observation that the quantity of type IV collagen present in the basal lamina of induced carcinomas is significantly less than that observed in normal epithelial basal lamina may indicate evidence of decreased synthesis of this substance or, alternatively, evidence of a greater rate of enzymatic degradation in tumours.

It has been reported that laminin plays important roles in tumour cell differentiation, tumour invasion and metastasis, and in the release of type IV collagenase [6, 7, 42]. From *in vitro* studies, it has been shown that the attachment activity of metastatic tumour cells can be promoted by laminin and that the laminin-attached tumour cells show greater metastatic propensity [43]. Furthermore, in a study of the presence of type IV collagen in neoplasms, it has been suggested that the expression of type IV collagen is dependent on production and destruction, both of which are related to epithelial proliferation, enzyme (such as metalloproteinase) activity [44], and other systematic (e.g. hormonal) factors [45]. This indicates that the expression of basal lamina type IV collagen and laminin in experimental oral carcinomas may be associated with the inherent metabolic properties of tumour cells, and/or microenvironmental factors such as the inter-relationships of extracellular matrix enzymes and components. Elucidation of the exact mechanisms behind the quantitative changes in type IV collagen and laminin in experimental oral carcinoma clearly requires further investigation.

On the basis of our observations we conclude that type IV collagen and laminin appear to be principally confined to the lamina densa of normal rat tongue epithelial basal lamina, and carcinoma-associated basal laminae. Furthermore, ultrastructural immunocytochemical observation of normal stromal fibroblasts and tumour stromal fibroblasts also indicate that laminin epitopes or laminin-like epitopes can be detected in these cells. Moreover, quantitative immunocytochemical studies of type IV collagen and laminin in both normal mucosa and experimental oral carcinomas reveal significantly increased lamina densa laminin and decreased type IV collagen in the basal laminae of carcinomas. The quantitative differences observed in these basal lamina molecules may relate to the ultrastructural morphological changes seen (such as thickening) in tumour basal laminae. These observations have important implications regarding tumour cell behaviour, and may also be of use in determining the malignant potential of oral epithelial dysplasias. Further studies examining human biopsy material are currently being conducted in order to extrapolate our findings from the animal model to human tissue.

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