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# Oral cancer: Role of the basement membrane in invasion

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**Abstract**

Invasive growth of cancer cells is a complex process involving specific interactions between tumour cells and the orderly, integrated complexes of the extracellular matrix. Basement membranes have been proposed as one constituent of extracellular matrix which carries responsibility for regulating invasion and metastasis. Using a chemically induced rat tongue carcinoma model, it has been shown that components of the basement membrane and its overall structure are altered during tumour invasion, and methods have been developed to quantitate some of these differences. Since the basement membrane can be specifically characterized by its fibrous protein network of Type IV collagen and laminin, which is embedded in a heparan sulphate-rich proteoglycan matrix, these components have been targeted. In particular, the current paper presents results in the context of current concepts of early changes in neoplastic invasion of underlying connective tissues. In consequence, further elaboration of the underlying mechanisms of epithelial migration in oral cancer may allow an exploration of the use of alterations in expression of basement membrane components as prognostic indicators.

***Key words:*** Heparan sulphate, immunohistochemistry, laminin, oral carcinoma, proteoglycans, Type IV collagen.

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## Introduction

Oral cancer is one of the ten most common cancers in the world, and accounts for between three and five per cent of all cancers in Western countries.[1] The primary oral sites of occurrence include buccal mucosa, tongue, alveolus, palate, lip and floor of the mouth. The most common sites for metastases of oral cancer are the regional lymph nodes. Oral cancer assumes a major health problem in terms of patient morbidity and mortality. Overall, the five-year survival rate is approximately 50-55 per cent, and for Stage III and IV cancers 35 per cent and 20 per cent respectively.[2,3]

The majority of oral cancers are epithelial squamous cell carcinomas, characterized by strands, sheets or islands of epithelial cells invading the underlying connective tissues, and accompanied by a proliferating fibrous stroma of non-neoplastic origin. The invading neoplastic epithelium resembles the original stratified squamous epithelium to various degrees (the grading or 'degree of differentiation' of the tumour). Currently, the prognosis for patients with oral squamous cell carcinoma depends upon both histologic subtype (grade) and clinical extent (stage) of the tumour. Well-differentiated lesions generally have a less aggressive course than poorly-differentiated lesions. However, according to Burkhardt,[4] grading remains subjective, and has little meaning for comparative studies or for the prognosis of the individual patient. More recently, several studies have provided encouraging evidence for differences in matrix components to be correlated with clinical prognosis.

Liotta proposed that invading tumour cells traverse two types of extracellular matrix (basement membranes and interstitial stroma), and that altered biochemical reactions between normal cells and matrix in neoplasia influence tumour invasion.[5]

Basement membrane (basal lamina) is a tough elastic structure consisting of a dense meshwork of Type IV and VI collagens, heparan sulphate (HS) (perlecan) and chondroitin sulphate (ChS) proteoglycans (versican), and glycoproteins such as laminin, nidogen (entactin), thrombospondin, tenascin, and fibronectin which are variously located in the lamina lucida and lamina densa areas of the

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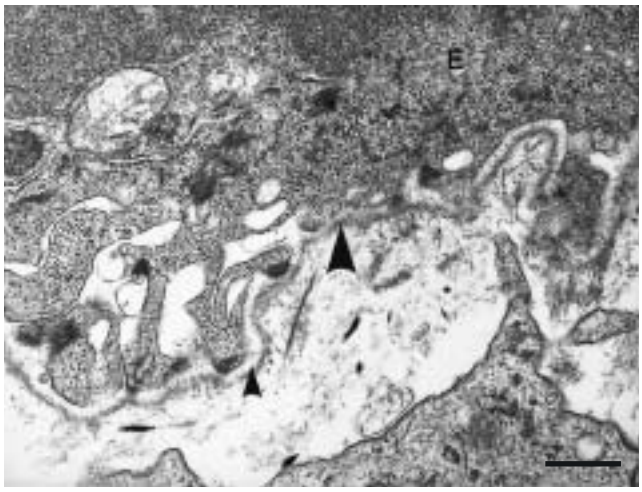


**Fig. 1.** – Transmission electron micrograph of normal rat tongue epithelium and lamina propria. Note basement membrane consisting of lamina lucida (small arrow) and lamina densa (large arrow). E=basal epithelial cell. [Bar=0.5 μm].

membrane (Fig. 1). Heparan sulphate proteoglycans (HSPG) are found tightly bound to all basement membranes. The presence of HSPG on the basolateral surfaces of epithelial cells (syndecan), or in association with such fibrous protein components of the basement membrane as laminin and Type IV collagen, has been established by using a variety of techniques.

Basement membranes appear to be crucial in tumour invasion and metastasis.[6] Loss of basement membrane has been associated with many types of carcinomas, and with tumour cells in lymph node and organ metastases. While invasive tumours may retain their ability to synthesize basement membrane constituents, assembly is often defective, and other loss may be due to decreased synthesis or increased turnover of basement membrane components stimulated by tumour cell-derived proteases. Hitherto, the authors have reported morphological changes in the basement membranes of those rat tongues where chemically-transformed cells are effecting early changes to the connective tissues. Using electron microscopy and immunohistochemistry, discontinuities, duplication, thickening, and intensive and/or absent staining have been identified.[7,8] The current study focuses on the components responsible for these changes.

Descriptions of specific changes in the various constituents of basement membrane have been few and most observations qualitative. More intensive staining of laminin and discontinuous staining of Type IV collagen have been demonstrated for transformed epithelial invasion.[8] The site of occurrence and functions of Type IV collagen relative to basement membrane are not well documented for either normal tissues or carcinomas. The glycoproteins thrombospondin and tenascin have also exhibited differences (enhanced staining) in tumour tissues.[9,10]

Degradation of HS in cultured tumour cells has been reported[11] and cell surface HS rich proteoglycans, such as syndecan or basement membrane proteoglycans, appear to be important in regulating the migration of transformed cells.[12]

At the ultrastructural level, there is little information describing the presence, distribution and quantitative aspects of basement membrane constituents for malignant neoplasms, in both human and experimentally induced animal carcinomas. According to Scully and Burkhardt, there are no reliable data on most basement membrane markers in potentially malignant oral lesions.[13] Lee suggested that the immunohistochemical localization of endothelial and basement membrane antigens could be better exploited as an objective means of identifying vascular invasion.[6] A major challenge in cancer research is to develop improved methods of predicting the invasiveness and subsequent metastatic propensity of a tumour. The techniques currently being reported are focused on characterizing and localizing actual changes in extracellular matrix components of basement membranes, on epithelial cells, and in the underlying connective tissues.

## Materials and methods

### *a) Animal carcinoma model*

Ethical approval for these experiments was granted by The University of Adelaide Animal Ethics Committee. A substantially refined model of 4-nitro-quinoline-1-oxide (4NQO) induced oral carcinoma in rats, originally described by Wallenius and Lekholm,[14] has been used as a source of non-neoplastic and neoplastic mucosa in these studies. Using this model the full range of clinical and microscopic features of dysplastic and malignant epithelium can be produced in the tongue by applying 4NQO to the dorsum of the tongue for periods up to 24 weeks.[7,8,15]

Histochemical and biochemical aspects of the model have previously been examined, using Alcian Blue-$MgCl_2$ critical electrolyte concentration methods, and analysis of proteoglycans extracted under associative and dissociative conditions from normal and carcinogen-treated rat mucosa.[16]

### *b) Immunohistochemistry*

The immunohistochemical identification of laminin and Type IV collagen in normal and cancerous rat tissues has also been established using light microscopy and the avidin-biotin peroxidase technique[8] and subsequently by both routine[7] and quantitative immunogold electron microscopy.[17,18] These latter techniques employed standard IgG-gold and Protein A-gold postembedding incubations.

### *c) Poly-L-lysine labelling of proteoglycans*

Identification of sulphated proteoglycans relies on electron microscopic localization using a gold-labelled poly-L-lysine.[19] Poly-L-lysine is a highly cationic molecule with affinity for anions. Particles of colloidal gold coated with poly-L-lysine serve as reliable markers for electron microscopy. This gold probe can be used at a range of pH values for differentially demonstrating anionic tissue components. To date, the published studies only suggest, albeit strongly, the localization of particular anionic components. By employing specific lyases, and chemical modifications of the substrates to eliminate glycosaminoglycan ligands in the tissue, the poly-L-lysine gold methods have been extended to identify particular proteoglycans. Specific identification of glycans such as variously sulphated ChS, sialated glycoproteins, and HSPG, was undertaken by means of appropriate enzyme predigestion (chondroitinase AC or ABC, neuraminidase, and heparitinase respectively). By varying the pH of poly-L-lysine colloidal gold binding conditions to generate high levels of hydrogen ion concentration, only sulphates are shown to bind to gold particles. Elimination by deamination of N-sulphated glycosaminoglycans (HS) was achieved by pre-treating sections with freshly prepared nitrous acid (18 per cent).[20] Some monoclonal antiproteoglycan antibodies (αsyndecan and αHS) have also been used at a light microscope level to confirm the localization in basement membranes. Anti-syndecan was prepared and characterized, and antiheparan sulphate (10E4) was purchased commercially.†

### *d) Methods of ultrastructural quantitation*

The method for quantitating poly-L-lysine gold markers in the rat model is based on analysis of immunogold labelling of basement membrane components in electron micrographs.[17,18] Statistical calculations have demonstrated that accurate quantitation can be achieved by particle counts/unit area of basement membrane in samples of five micrographs/animal at ×15 000 original magnification. Initial mathematical analysis of data was undertaken using the component of variance model. Final statistical analysis was achieved with programs 3V, 5V and 8V from the BMDP statistical package. This method of quantitation was used for routine analysis of both animal and human material.

## Results

### *Morphological changes in basement membrane*

Examination of the basal lamina around neoplastic epithelial tissues revealed features in some areas analogous to normal ultrastructural morphology. However, in other areas, discontinuities and prominent focal thickening, characterized by increased lamina densa-like material, were observed.[7]

### *Laminin*

An increased intensity of immunogold labelling for laminin was seen in the lamina densa of tumour basement membrane in all specimens. Gold particles were also observed in the tumour stroma, in some carcinoma cells, and in stromal fibroblasts. Statistical analysis of quantitative data indicated that the label density of lamina densa laminin [number of gold particles per $\mu m^2$ (area) or per μm (length)] in the experimental oral carcinomas was significantly higher than that of the untreated animals ($p<0.01$).[8,17]

### *Type IV collagen*

Immunogold labelling for epithelial basal lamina Type IV collagen in carcinomas revealed variable expression, principally in the lamina densa at a level of intensity less than that observed for laminin in carcinomas. Occasional gold particles were evident in the tumour stroma. The label density of lamina densa Type IV collagen in the experimental oral carcinomas was significantly lower than that of the untreated group ($p<0.01$).[8,17]

### *Proteoglycans*

Adjustment of pH from 1 to 2.5 and pretreatment with neuraminidase eliminated staining of sialated glycoproteins. In order to differentiate HS from ChS in the basement membrane of rat tongue mucosa, heparitinase, nitrous acid, and chondroitinase ABC were used. After pretreating tissue sections with these specific enzymes and following deamination, labelling of basal lamina was substantially attributed to HS. In comparison with normal tissues, neoplastic tissues exhibited less dense, more scattered labelling of gold particles in the basal lamina, and in the tumour stroma at pH 1.0 (Fig. 2). Quantitative analysis indicated that the label density was significantly lower than that of normal mucosa ($p<0.01$).[18]

## Discussion

Several studies including those of the authors have focused on morphological staining patterns for Type IV collagen and laminin in neoplasms at the light and electron microscopic level.[6,8,17,21]The focal thickenings and discontinuities noted in the present studies in predictive induced tongue carcinomas in rats have also been reported for human carcinomas and other induced animal oral carcinomas.[7,22,23] According to Tsujioka *et al.*, the thickening may

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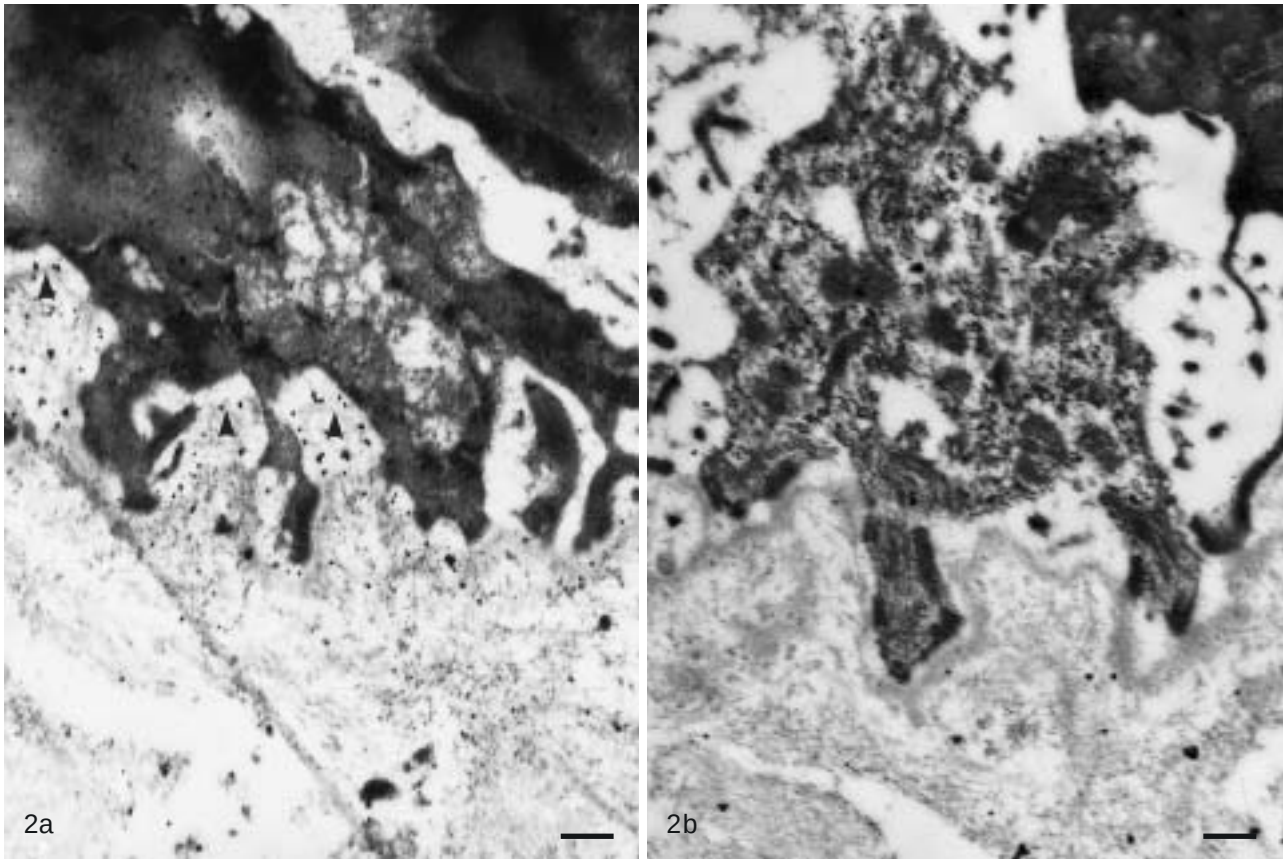


**Fig. 2.** – Poly-L-lysine colloidal gold stained postembedded sections of rat tongue before (a) and after (b) heparitinase treatment to show specific elimination of heparan sulphate substrate. Note label on the basement membranes of induced rat carcinomas (arrows) showed a reduction of labelling of 60 per cent.[18] [Bar=0.2 μm].

represent regeneration of basal lamina.[23] Quantitatively, the ultrastructural data obtained in the present studies shows that the amount of laminin and Type IV collagen in the basal lamina of normal rat epithelium and in carcinomas derived from this tissue varies.[17] The increased expression of laminin in tumour basal lamina may represent an increase in its synthesis in tumour tissue and, in turn, may account for the increased thickness of basal lamina observed in our previous tumour studies[7] and by others.[23] Conversely, the quantity of Type IV collagen present in the basal lamina of induced carcinomas is significantly reduced over normal epithelial basal lamina. Both a decreased synthesis, or a potent rate of enzymatic degradation in tumours are thought to be responsible. Collagenase and the mucosal mast cell chymo-tryptase, RMCPII, are two likely candidates. These observations have important implications regarding tumour cell behaviour, and may also be of use in determining the malignant potential of oral epithelial dysplasias.

In localization studies of acidic glycoconjugates, the use of enzyme elimination and pH differentiation is a relatively recent ultrastructural histochemical method. Furthermore, the expression of non-cell-associated HSPGs in basal lamina from both normal oral mucosa and induced oral carcinomas has been demonstrated as diagnostic of early neoplastic changes.[18]

The observation of significantly less labelling of HS in carcinoma tissues may be related to the low metastatic propensity of these experimentally-induced well-differentiated lesions.[15] The diversity of reported roles for HSPG in growth factor presentation, differential charge distribution and other extra-cellular interactions suggests that caution is needed in speculating about their role in neoplastic proliferation, invasion and metastasis. Nevertheless, the present findings suggest that not only are the expressions of transmembrane proteoglycans, like syndecan, imputed but structural extracellular molecules can reflect the status of tumour growth.

The use of ultrastructural immunolabelling as a research and prognostic/diagnostic tool has become more popular due to the introduction of simpler methods and more specific antibodies. The technique can combine precise morphological localization with highly specific antigenic epitope detection. Herrera had suggested that quantitation of antigenic determinants amplifies the usefulness of ultrastructural immunolabelling by providing an objective means of evaluating antigenic expression, and a source for comparison of specific antigenic epitopes in tumours showing similar histological

appearances.[24] This author proposed that future applications of immunoelectron microscopy to diagnostic surgical pathology would address a variety of controversial issues pertaining to tumour diagnosis. This notion, together with improved antigen detection methods,[25] provides precise quantitative localization of collagen types, laminin, and polyanions such as HSPG.

Further studies, currently in progress, offer the real potential for a more detailed understanding of neoplastic phenomena, such as invasion and metastasis in other tumours. The histopathological armament is now augmented with prognosticators other than transformation antigens *per se*, resulting in a specific emphasis on the matrix within which neoplastic invasion occurs. This focuses on the pivotal need for transformed cells to breach the basement membrane before they can metastasize to other sites.


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