Pergamon

Oral Oncol, Eur J Cancer, Vol. 32B, No. 1, pp. 8–13, 1996

Printed in Great Britain
0964–1955/96 $15.00+0.00

0964–1955(95)00064–X

Papers

Identification of Basal Lamina Acidic Glycoconjugates, Particularly Heparan Sulphate Proteoglycans, Using a Poly-L-lysine–Gold Probe in Induced Oral Carcinomas

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Acidic glycoconjugates represent the major non-fibrous macromolecular components that form the extracellular and cell-associated matrices of all animal tissues. The constituent molecules are principally structural glycoproteins and proteoglycans. While their protein component is determined by gene pools, it is the polyanionic (acidic) nature of the polysaccharides, determined by their degrees of carboxylation and sulphation, which confers both functional and diagnostic status on these molecules. Sulphated glycoconjugates in the basal laminae have been reported to play a role in tumour invasion and metastasis. In this study, we used cationic colloidal gold together with transmission electron microscopic methods to compare the expression of acidic glyconconjugates in the basal lamina of both normal rat tongue mucosa and experimentally induced oral carcinomas. Results indicated that heparan sulphate rich glycoconjugates were predominant and were mostly confined to the lamina lucida of the basal lamina in normal oral mucosa. Conversely, observation of basal laminae associated with induced carcinomas showed less intense and more widely dispersed gold labelling for heparan sulphate. The observed differences in gold labelling may reflect modified metabolism of sulphated glycoconjugates or result from the action of degradative enzymes in the induced tumours.

Keywords: acidic glycoconjugates, basement membrane, colloidal gold, heparan sulphate, proteoglycans, rat, oral carcinoma

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INTRODUCTION

Cellular and biochemical events in tissues are dominated by the presence of the structural glycoproteins and the proteoglycans which comprise the extracellular matrix. The net negative charge of acidic glycoproteins and glycosaminoglycan chains of all proteoglycans results in a variety of functions and is determined by the carboxyls (COO^-) on the constituent sialic and hexuronic acids, respectively, together with the various degree and position of the sulphates. These differences can be manipulated to provide reactive anionic groups for histolocalisation [1]. The distribution of these molecules has been related to cellular maturation and to disease processes, such as neoplasia [2]. They are variously arranged in tissues as glycoconjugates and their localisation has previously been carried out using a variety of techniques, including biochemical, immunohistochemical and histochemical methods [1, 3–5]. These methods and the biology of these molecules have been reviewed by Thiéry and Ovtracht [1] and Ruoslahti [6] and, as described by them, localisation of glycoconjugates using such techniques in various tissues has produced equivocal results.

Basal lamina is a specialised extracellular structure rich in both carboxylated and sulphated glycoconjugates, especially heparan sulphate, and it appears to play an important role in the processes of neoplastic invasion and metastasis [7–11]. Whereas neoplastic invasion has been reported to be glycoconjugate dependent, a variety of modifications in extracellular matrix components, including heparan sulphate content, have also been reported [11].

Using biochemical methods, alterations in some acidic glycoconjugates in neoplastic tissues have been reported by

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Received 15 Dec. 1994; provisionally accepted 5 Jan. 1995; revised manuscript received 6 Oct. 1995.

previous investigators [12–14], but the distribution of acidic glycoconjugates in the basal lamina of neoplastic tissues, including oral carcinoma, is poorly understood. In this study, we have modified the cationic colloidal gold label conjugation procedure (with poly-L-lysine) developed by Skutelsky and Roth [15], by employing combinations of specific glycan lyases to identify acidic glycoconjugates in the basal laminae of normal rat mucosa and experimentally induced oral carcinomas. By focusing on the specific elimination of: (a) the anionic charges on sialo-glycoproteins which are responsible for gold-particle binding using neuraminidase; (b) the α-*N*-acetyl-D glucoaminide linkage of heparan sulphate using heparitinase; (c) the chondroitin and dermatan sulphates using chondroitin ABC lyase; and (d) by selectively ionising the substrates under increasing pH, we have differentially localised basal lamina components on ultrathin sections. The distribution of acidic glycoconjugates in the basal laminae of normal rat mucosa epithelium and induced oral carcinomas is described.

MATERIALS AND METHODS

Tissue source and preparation

Tissue specimens were obtained from five normal female Wistar rat tongues and five 4-nitroquinoline 1-oxide (4NQO)-induced rat tongue carcinomas. The induction of experimental oral carcinomas and the methods of tissue processing were similar to the procedures described by Jiang *et al.* [16]. Briefly, 10 µl of 0.5% 4NQO in propylene glycol was applied three times per week for 24 weeks to the dorsal tongue surface of anaesthetised rats to induce carcinomas. After sacrifice, suitably sized (approximately 1 mm^3) tissue specimens were fixed by immersion in 0.25% glutaraldehyde–0.15% picric acid–5% sucrose in 0.1 mol/l phosphate buffer (PB), pH 7.2, for 1 h and dehydrated through a series of graded ethanols for 1 h. Tissues were infiltrated and embedded in L.R. White resin (The London Resin Company, London, U.K.) with the manufacturer's accelerator at room temperature for 5–6 h. Ultrathin sections were cut from tissue blocks and mounted on nickel grids ready for staining with poly-L-lysine–gold complexes.

Preparation of poly-L-lysine–gold complex

A colloidal gold solution with a gold particle diameter of approximately 15 nm was prepared by reducing 1% chloroauric acid ($HAuCl_4$) (Ajax Chemicals, Sydney, Australia) with 1% tri-sodium citrate $2H_2O$ (Merck, Dornstad, Germany) using the method described by Slot and Geuze [17]. The preparation of the poly-L-lysine–gold complex was based on the procedure described by Skutelsky and Roth [15]. Briefly, the optimal amount of poly-L-lysine (MW = 39 800) (Sigma, St. Louis, Missouri, U.S.A.) required to stabilise the colloidal–gold solution was determined by the salt flocculation test. The poly-L-lysine–gold complex was obtained by mixing the colloidal gold sol with the optimal amount of poly-L-lysine. The complex was differentially pelleted by two centrifugations at 60 000*g* each for 45 min at 4°C in fixed angle tubes and stored ready for use. The first centrifugation yielded a pellet of complexed gold with uncomplexed material remaining bound to the centrifuge tube wall.

Specimen incubation with the poly-L-lysine–gold complex

The effect of specific glycan lyase pretreatment of sections on the binding of poly-L-lysine–gold and the role of pH were assessed by incubating normal mucosa specimens under the following protocols:

Ultrathin sections mounted on grids were incubated by sequential inversion on to one drop of the following solutions:

(1) 0.15 mol/l glycine in phosphate-buffered saline (PBS) for 15 min;
(2) 1:10 poly-L-lysine–gold complex diluted with PBS containing 0.1% bovine serum albumin (BSA) and 0.02% polyethylene glycol at pH 7.0, 6.0, 5.0, 4.0, 2.5 and 1.0 for 1 h each;
(3) three PBS rinses for 5 min each;
(4) counterstained with 2% aqueous uranyl acetate for 2 min and Reynold's lead citrate for 30 s.

Having established the optimum pH, sections derived from carcinoma specimens, as well as freshly cut normal mucosa specimens, were simultaneously incubated using the above protocol at pH 1.0 only.

Control and glycan lyase discriminatory incubations

Sections were preincubated with the following solutions prior to incubation with the poly-L-lysine–gold complex:

(1) neuraminidase (1 U/ml in PBS, pH 6.0, at 37°C) (Sigma) for 1 h;
(2) neuraminidase (1 U/ml in 0.05 mol/l PBS, pH 6.0, at 37°C) and heparitinase (1 U/ml, 1 µmol/l calcium acetate) (Seikagaku, Tokyo, Japan) for 1 h each;
(3) neuraminidase (1 U/ml in 0.05 mol/l PBS, pH 6.0, at 37°C) and chondroitinase ABC (1 U/ml in PBS) (Seikagaku) for 1 h each;
(4) heparitinase (1 U/ml, 1 µmol/l calcium acetate) (Seikagaku) for 1 h;
(5) non-labelled poly-L-lysine for 1 h.

Additional control sections were incubated with colloidal gold solution only for 1 h. All stained sections were examined in a Jeol 100S transmission electron microscope.

Quantitative analysis

Quantitative analysis was carried out following the procedure described by Jiang *et al.* [16]. In brief, tissue samples consisted of up to five normal rat tongues and five 4NQO-induced rat tongue carcinomas for each analysis. Tissue blocks were randomly selected from tissue block pools. One ultrathin section per block was randomly selected for staining. Five electron micrographs were randomly selected for electron microscopic examination. Since staining was a post-sectioning event and is, in consequence, a two-dimensional surface phenomenon, the accuracy of gold particle counting from photomicrographs was not affected by variables in penetration of the colloidal gold into the tissue block, nor variation in the section thickness. Only substrate, exposed to the reagents, is available for elimination, blocking or staining. The label density of heparan sulphate was represented by the number of gold particles per unit area. All data were subjected to significance testing by analysis of variance, or by the Mann–Witney U-test to account for any differences in standard errors.

RESULTS

Effect of pH and enzyme pretreatment of sections

Labelling by gold particles was seen on all normal tissue sections treated at the different pH levels (pH 6.0, 5.0, 4.0, 2.5

and 1.0). No gold particles were seen on tissue sections treated at pH 7.0. At pH 6.0, 5.0 and 4.0, numerous gold particles were seen in the epithelial cells themselves, in the epithelial intercellular spaces, basal lamina regions and connective tissues. At pH 2.5 and pH 1.0 (Fig. 1), while gold particles were mainly distributed along the basal lamina regions, some gold particles were observed in the epithelial intercellular spaces and in the underlying connective tissues.

After pre-incubation of tissue sections with neuraminidase at pH 2.5 and pH 1.0, there was an obvious reduction of gold particles over basal lamina regions (Fig. 2). Similarly, labelling

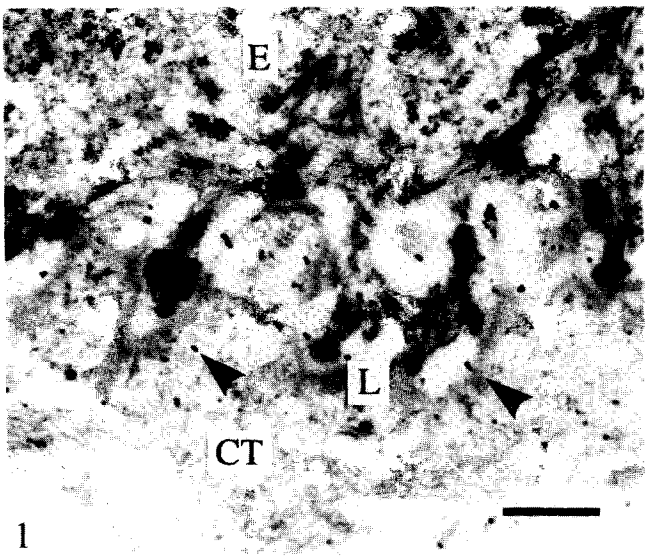

Fig. 1. Rat normal oral mucosa. Stained with poly-L-lysine–gold at pH 1.0. Electron micrograph showing gold particles (arrows) distributed in basal lamina (L) regions. A number of particles are also seen in the connective tissue (CT) adjacent to the basal lamina. E = epithelial cell (bar = 0.2 μm).

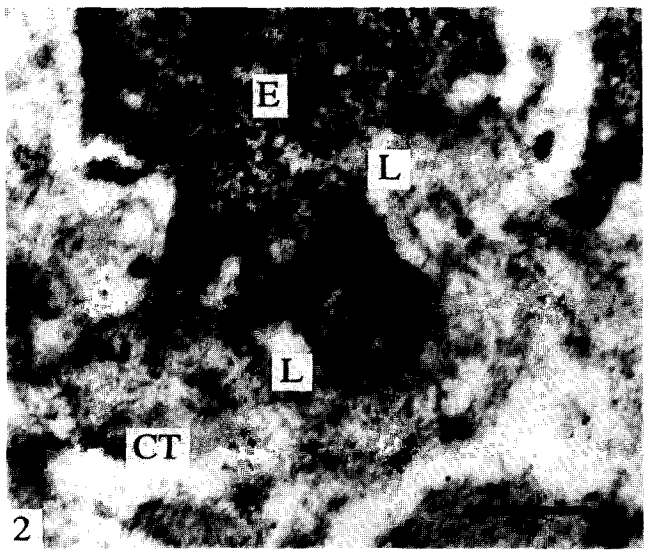

Fig. 2. Rat normal oral mucosa. Stained with poly-L-lysine–gold at pH 1.0. Pretreatment with neuraminidase. Electron micrograph demonstrating an obvious decrease in gold-labelling along the basal lamina (L) and in the adjacent connective tissue (CT). (Compare with Fig. 1.) E = epithelial cell (bar = 0.2 μm).

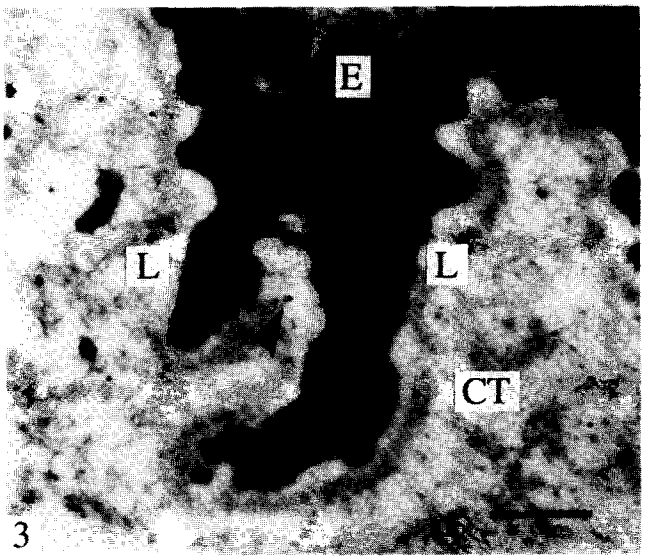

Fig. 3. Rat normal oral mucosa. Stained with poly-L-lysine–gold at pH 1.0. Pretreatment of section with neuraminidase plus heparitinase. Electron micrograph indicating occasional gold particles (arrows) along the basal lamina (L). E = epithelial cell, CT = connective tissue (bar = 0.2 μm).

of the intercellular spaces and the connective tissues showed an obvious decrease. Almost all basal lamina labelling was abolished after pre-incubation of tissue sections either with heparitinase alone, or with heparitinase following neuraminidase (Fig. 3). However, some gold particle labelling still appeared in the underlying connective tissue. No marked changes in the intensity of gold particle labelling was observed along basal lamina regions after predigestion of tissue sections by chondroitinase ABC. No gold particle labelling was seen in tissue sections incubated with non-labelled poly-L-lysine or colloidal gold alone.

Normal and neoplastic tissue analysis

In normal rat tongue oral mucosa, consistent gold particle labelling was evident in the basal laminae of all tissue specimens treated at pH 1.0. It was noted that gold particles were mainly located to the lamina lucida of basal laminae (Fig. 4). Some gold particles were also seen in the adjacent connective tissues. 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. 5). Quantitative analysis indicated that the label density on basal lamina of 4NQO-induced oral carcinomas was significantly lower than that of normal rat oral mucosa ($P < 0.01$; observer group 1) ($P < 0.05$; observer group 2) (Table 1).

DISCUSSION

Glycoconjugates as components of the basal lamina

The acid components of molecules classified as glycoconjugates are represented by polysaccharide structures, such as the unbranched sulphated glycosaminoglycans (heparin, heparan sulphate, chondroitin sulphates and keratan sulphate), as well as the simple and complex branching carbohydrates of glycoproteins. The heparan sulphate chains are the important acidic structures of various basal laminae which, in these tissue compartments, contribute to molecular organisation, basal

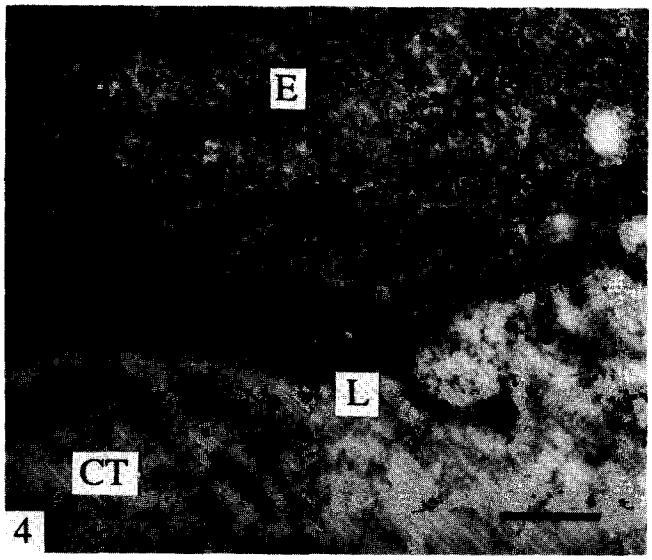

Fig. 4. Normal rat tongue mucosa. Stained with poly-L-lysine–gold at pH 1.0. Electron micrograph indicating a labelling pattern similar to that seen in Fig. 1. E = epithelial cell, L = basal lamina, CT = connective tissue (bar = 0.2 μm).

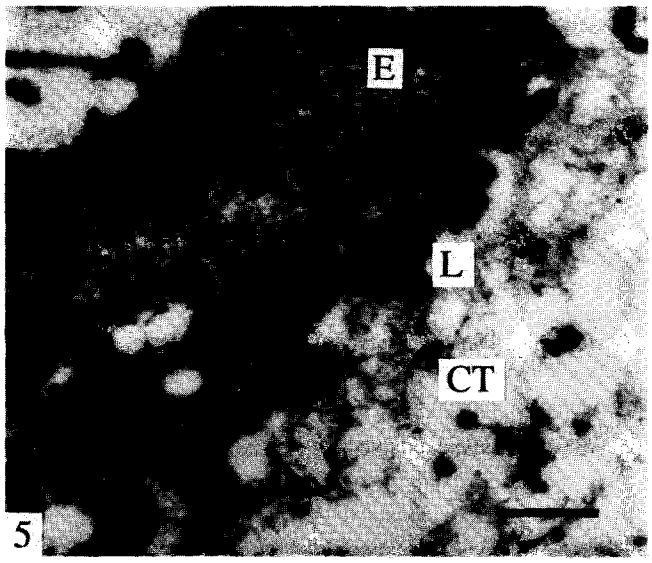

Fig. 5. Induced rat tongue carcinoma. Stained with poly-L-lysine–gold at pH 1.0. Electron micrograph showing a paucity of gold particles (arrows) along the basal lamina (L). E = epithelial cell, CT = connective tissue (bar = 0.2 μm).

lamina permeability and tumour invasion and metastasis [7]. While the predominant heparan sulphate proteoglycan in the basement membrane, specifically in Englebreth–Holm–Swarm murine tumours, has been known since the early 1980s as perlecan [18], the other distinct heparan sulphate-linked gene pools that can be localised to this site [19] can even include transmembrane structures such as syndecan. Chondroitin sulphates are also reported to be localised in the basal lamina of some tissues [20]. Some confusion may arise from the immunolocalisation of certain heparan sulphate proteoglycans. For example, antibodies to the terminal third of the A chain of laminin will cross-react with the two regions of close homology in the perlecan core [21]. Thus, even at the ultrastructural level, equivocation about this can arise. While this report does address the presence of heparan sulphate proteoglycan, it does not focus on the functional species of this glycoconjugate.

Table 1. Mean and median number of gold particles per μm² of basal lamina from normal rat tongue and rat tongue carcinoma

	Normal rat tongue	Carcinoma
Observer group 1		
Mean density of gold particles (±S.D.)	42.50 (±18.35)	13.76 (±11.59)*
n	50	50
Observer group 2		
Median density of gold particles (±S.D.)	32.01 (±22.07)	11.21 (±5.81)**
n†	14	14

n, number of electron photomicrographs examined.
*$P < 0.01$ between groups (observer group 1) (analysis of variance).
**$P < 0.05$ between groups (observer group 2) (Mann–Witney U-test).
†The number of photomicrographs selected for counting comprised 14 randomly selected photomicrographs from the 50 analysed by observer group 1.

Hitherto, interpretation of demonstrations of such acidic glycoconjugates in a number of tissues using various histochemical methods at the optical microscopic level has also been equivocal. Of these methods, the differential binding of cationic chromophores to the polyanionic carboxyls and sulphates is a common and reliable feature. A range of similar approaches which use cationic reagents has been used at the ultrastructural level [1, 15]. In extending these approaches, we have incorporated the use of enzyme elimination and pH differentiation with one relatively new ultrastructural histochemical method [15] to localise acidic glycoconjugates in the basal lamina of normal rat tongue and in induced oral carcinomas.

Poly-L-lysine conjugated colloidal gold: differential binding to basal lamina

The technique relies on the availability and degree of ionisation of acidic groups on carbohydrate side chains for binding to electron dense colloidal gold. Colloidal gold was rendered cationic with poly-L-lysine. This, in combination with the elimination with specific glycan lyases to discriminate specific polyanionic substrates, was applied to both normal and neoplastic tissues. Compared with previous studies, where high molecular weight poly-L-lysine (>300 kDa) was recommended [22, 23], we have generated satisfactory labelling with low molecular weight poly-L-lysine (39.8 kDa). The glycoconjugate structures on which we have focused include the sialic acid (*N*-acetyl neuraminic acid) of the sialioglycoproteins, as well as the heparan and chondroitin sulphates (including dermatan sulphate) of the glycosaminoglycan side-chains. The binding sites for cationic gold on sialoglycoproteins is the sialic acid. In contrast, the glycosaminoglycans are substantially devoid of sialic acid, and rely on their sulphate content for gold labelling.

The glycan lyases used in this study were neuraminidase,

heparitinase and chondroitinase ABC. Neuraminidase is a specific enzyme for the digestion of those glycoproteins where the sialic acid is in the open chain form of the anomer. The heparitinase catalyses the eliminative cleavage of the μ-*N*-acetyl-D glucosaminide linkage of the heparan sulphates but not heparin. Chondroitinase ABC shows specificity towards the cleavage of chondoitin sulphates as well as dermatan sulphate. Further justification for the conditions chosen for this study lies with the relative dissociation constants (p*K*) which determine ligand binding between the substrate molecules and the polycationic poly-L-lysine colloidal gold. Only the strongly sulphated group in the glycoconjugate should be ionized at the very low pH of 1.0 [1]. This means that despite any elimination of the anionic carboxyls of sialic acid from tissue sections resulting from pretreatment with neuraminidase, subsequent exposure to cationic colloidal gold at the low pH of 1.0 should be irrelevant to the selectivity of binding. At this high level of hydrogen ion concentration, only sulphates would be expected to bind the gold particles.

Nevertheless, it is interesting that in the present study a decrease in labelling was observed in basal lamina regions at pH 1.0 after the predigestion of tissue sections with neuraminidase. In contrast to determinations based on pH, this observation emphasises the importance of dissociation constants (p*K*) rather than pH in the interpretation of the form of the polyanion. Indeed, the reported p*K* values, which range from 3.3 to 2.4 between the available carboxyls of the constituent glucuronic acids of glycosaminoglycan repeats and the sialic acids of the glycoproteins, would yield only some dissociated carboxyls which would, in turn, be unavailable for reaction with the colloidal gold. Of more importance in this study is the substantial loss of gold labelling at pH 1.0 in basal lamina on tissue sections after heparitinase digestion. This represents a loss of heparan sulphate. The quantitative differential between the number of gold particles on neuraminidase-treated and heparitinase-treated basal laminae is likely to represent the difference between non-hydrolysable sialic acid anomer and enzyme-susceptible glycoprotein substrate irrespective of pH. Any residual difference in gold may be accounted for by chondroitin sulphate.

In order to differentiate heparan sulphate from chondroitin sulphate in the basal lamina of rat tongue mucosa, heparitinase and chondroitinase ABC were used. After the predigestion regime of tissue sections with these specific enzymes, labelling of basal lamina was substantially attributed to heparan sulphate.

The reported location and specificity of heparan sulphate in the basal lamina of tissues seems to be dependent on the differences in molecular structure, types of antibody and detection methods used [5, 24–26]. Embedding and fixation techniques may also contribute to this equivocation. In the present study, a relatively consistent localisation of labelling was seen in all normal tissue specimens. While heparan sulphate appeared to be mainly located in the lamina lucida of basal lamina of normal rat tongue mucosa, it is possible that its protein core could be situated in the lamina densa, with its sulphated glycosaminoglycan side-chains extending into both sides of the lamina densa [27]. Gold particles were found on both sides of the lamina densa.

Basement membrane heparan sulphates and neoplasms

The role of heparan sulphate has been related to its distribution and molecular structure [28]. Furthermore, in malignant neoplasms, it has been thought that qualitative and quantitative changes in heparan sulphate proteoglycans are associated with the invasive and metastatic properties of tumours [7, 13, 14, 28, 29]. For instance, a heparan sulphate with a high degree of sulphation has been detected in highly metastatic tumours [13]. Mangakis *et al.* [12] reported that a high level of heparan sulphate was found in breast tumours and it was indicated that the patients with high levels of heparan sulphate had a poor prognosis. In other studies, Esko *et al.* [11] measured the tumorigenicity of proteoglycan-deficient Chinese hamster ovary cell mutants in nude mice. Mutants with less than 15% wild-type level of proteoglycan synthesis did not produce tumours and, in particular, cells with defective heparan sulphate proteoglycan synthesis did not form tumours. Inki *et al.* [30], more recently emphasised the importance of site and molecular specificity of heparan sulphate proteoglycan in determining clinical outcomes of squamous cell carcinomas. They reported a positive correlation between the transmembrane heparan sulphate hybrid, syndecan, and a favourable prognosis for squamous cell carcinoma.

In our present study, we compared the expression of non-cell-associated sulphated glycoconjugates (heparan sulphate) in basal lamina between normal oral mucosa and induced (well-differentiated) oral carcinomas and report that there was significantly less labelling in carcinoma tissues. It is known that our experimentally-induced oral carcinoma is characterised by a low metastatic propensity [31]. We propose that the expression of lowly sulphated glycoconjugates (heparan sulphate) in four NQO-induced oral carcinomas may relate to the low metastatic behaviour of this experimental oral carcinoma. Given the apparent, wide disparity in observations about the role of heparan sulphate proteoglycan in neoplastic proliferation, invasion and metastasis, we would need to be cautious about allocating mechanisms to our findings. First, the polymorphism of heparan sulphate motifs along the polysaccharide chain implies a spectrum of multifunctional roles that will include growth factor binding, e.g. FGF [32], through to prothrombin binding [33] and angiogenesis [34]. While the former is implicated in a very complex way with cell surface heparan sulphates, say syndecan, the latter will be substantially associated with vascularisation. Other than being a molecular component, integral with the physical structure of basement membrane architecture, these heparan sulphate proteoglycans may play an important role in inhibiting, or at least regulating, membrane turnover. Walsh *et al.* [35, 36] have reported the binding and inhibition of human leucocyte elastase to a cryptic motif on a heparatinase-derived low molecular weight fraction. The presence or absence of such a binding site in a basement membrane heparan sulphate oligosaccharide would be pivotal in the regulation or otherwise of basement membrane organisation without evoking cellular involvement, i.e. cell-surface receptors. The presence of mammalian heparitinases, especially from highly metastatic adenocarcinomas would mitigate in favour of this mechanism. An alternative role for a modified "non-cell-surface" heparan sulphate proteoglycan in the basal lamina is one of interaction with other membrane components. Both a degradative loss or inhibited synthesis of any one of a number of matrix components will results in faulty extracelluar matrix. Examples of this come from other connective tissue pathologies, such as early experimental osteoarthritic cartilage or periodontal disease, gingivae. Again, proliferation and invasion, but not necessarily metastasis, could result.

The mechanisms that involve heparan sulphate in regulating neoplastic development and spread remain poorly understood. Nevertheless, this report emphasises that not only are the expressions of transmembrane proteoglycans like syndecan imputed, but structural extracellular molecules can reflect the status of tumour growth.

Using the poly-L-lysine–gold technique, together with morphometry, a comparative quantitative analysis of these sulphated glycocomponents in normal oral mucosa and induced oral carcinomas has been possible. Such studies, currently in progress, offer the real potential for a more detailed understanding of the correlation between neoplastic phenomena, such as invasion and metastasis in other tumours, and the molecular composition of basal lamina in human pathological material.

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Acknowledgements—This study was supported by grants from the Australian Research Council, the University of Adelaide, and The Australian Dental Research Fund.