Structural differences between heparan sulphates of proteoglycan involved in the formation of basement membranes *in vivo* by Lewis-lung-carcinoma-derived cloned cells with different metastatic potentials

Hayao NAKANISHI,*¶ Kayoko OGURI,* Keiichi YOSHIDA,† Naoki ITANO,* Keizo TAKENAGA,‡ Takashi KAZAMA,§ Aichi YOSHIDA|| and Minoru OKAYAMA***

*Clinical Research Institute, National Nagoya Hospital, Nagoya 460, †Tokyo Institute of Seikagaku Corporation, Tokyo 189,

‡Department of Chemotherapy, Chiba Cancer Center Institute, Chiba 280, §Department of Dermatology,

Niigata University School of Medicine, Niigata 951, and ||Department of Pathology,

Kagoshima University School of Medicine, Kagoshima 890, Japan

This study addresses the characterization of heparan sulphates of the basement-membrane proteoglycans in tumour formed after the subcutaneous implantation of Lewis-lung-carcinoma-derived different metastatic clones (P29, LM12-3 and LM60-D6 clones with low, medium and high metastatic potentials respectively). Heparan sulphate proteoglycans (125–158 μ g of hexuronate/g dry weight of tissue) were isolated from chondroitin ABC lyase digests of a proteoglycan fraction obtained after DEAE-Sephacel chromatography of tissue extracts. The proteoglycans were separated into three molecular species by Sepharose CL-4B chromatography followed by CsCl-density-gradient centrifugation: large proteoglycans with an estimated M_r of 820 000–130 000, which consisted of two components with low (< 1.34 g/ml; PGII-M) and high (> 1.37 g/ml; PGII-B) density, and a small proteoglycan with an M_r of less than 80 000 (PGIII). Of these, only the PGII-M proteoglycan (34–37 μ g of hexuronate/g dry weight) reacted with the antiserum against proteoglycan of Engelbreth–Holm–Swarm-tumour basement membrane, and represented, therefore, a basement-membrane proteoglycan. Digestion with heparan sulphate lyases I and II of the heparan sulphates (M_r 36 000) from the PGII-M proteoglycan of the three tumours resulted in almost complete depolymerization to give six unsaturated disaccharides identified as 2-acetamido-2-deoxy-4-*O*-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-D-glucose, 2-acetamido-2-deoxy-4-*O*-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-D-glucose, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-2-*O*-sulpho- α -L-threo-hex-4-enopyranosyluronic acid)-D-glucose and 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-2-*O*-sulpho- α -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose. Comparison of the relative amounts of these disaccharides produced from the three tumour-derived heparan sulphates demonstrated that the degree of sulphation of the heparan sulphates correlated with the degree of morphological organization of the tumour basement membranes; the heparan sulphate from the more highly metastatic tumour with more highly organized basement membrane exhibited a higher degree of overall sulphation along the glycosaminoglycan chains, which was due to an increased content of the three repeating disaccharides having 6-*O*-sulphated glucosamine residues.

INTRODUCTION

Basement membranes are extracellular matrices that mediate cell attachment, separate tissue compartments and maintain tissue architecture throughout adult life. During embryonic development and adult tissue remodelling, the basement membranes serve as inductive interfaces and mediate morphogenetic interactions between epithelial cells and mesenchymal cells [1–3]. The basement membrane formed by tumour cells (tumour basement membrane) mediates the neoplastic organization of epithelial tumours [4,5]. In various carcinomas, the tumour basement membrane shows a variety of structural abnormalities, such as thinning, multilayering, discontinuity and total loss [6,7]. There are several reports that the loss of tumour basement membranes, probably caused by the loss of the ability of tumour cells to synthesize or secrete basement-membrane components or the acquisition of enzymic activity to degrade these materials, is

associated with invasion, and the tumour basement membrane acts as a barrier against tumour invasion [8,9]. However, there are other lines of evidence that many invading carcinomas retain their basement membrane in both primary and secondary sites [10,11], and these authors have claimed that the loss of the tumour basement membrane is not always associated with invasion [10,11]. Moreover, in some tumours, the tumour basement membrane appears to mediate tumour invasion and metastasis [12–14]. Thus, at present, the role of the tumour basement membrane in neoplastic development is controversial.

A variety of structural abnormalities of tumour basement membrane may be attributable to (1) an imbalance of synthesis and/or secretion of the constitutive molecules, i.e. type IV collagen, glycoproteins such as laminin, entactin and fibronectin, and heparan sulphate proteoglycan [15], (2) a defect in the assembly of these macromolecules due to molecular alteration [16] and (3) local degradation of these molecules. However, little

Abbreviations used: unsaturated disaccharides are defined in the text; EHS, Engelbreth–Holm–Swarm.

¶ Present address: Laboratory of Pathology, Aichi Cancer Center Research Institute, Nagoya 464, Japan.

*** To whom correspondence should be sent.

is known about this subject. There are several reports indicating that a poor formation of basement membrane by transformed cells is correlated with the reduced sulphation of heparan sulphate chains of basement-membrane proteoglycan [17,18]. Reduced sulphation of heparan sulphate chains is often induced in association with the oncogenic transformation of a variety of cells, resulting in an impairment of the ability of the cells to interact with other matrix constituents [19,20]. All these results suggest that some alterations of the isomeric structures of heparan sulphates of basement-membrane proteoglycan result in the impaired interaction with other basement-membrane constituents [17,18] in which heparin- and/or heparan sulphate-binding domains have been identified [21–23].

The above considerations prompted us to study the correlation between the morphology of the basement membrane formed by tumour cells and the molecular characteristics of its constituents. For this purpose we chose mouse Lewis-lung-carcinoma (3LL)-derived cloned cell lines with different metastatic potentials, several properties *in vitro* of which, correlating with their metastatic potentials, have previously been investigated [24,25]. We recently demonstrated that the more highly metastatic clone formed a more highly integrated basement membrane *in vivo* than the low metastatic one [13], that is, cloning of the cell lines in relation to their spontaneous metastatic potentials resulted in selection of the ability to form a basement membrane *in vivo*. In the present investigation we have focused on the relationship between the morphology of the basement membrane formed *in vivo* by Lewis-lung-carcinoma-derived cloned cells and the isomeric structure, especially the sulphation pattern, of the heparan sulphate chains of the proteoglycan that was found actually to be involved in construction of the basement membrane.

EXPERIMENTAL

Materials

Inbred C57BL/6 male mice, 6–8 weeks old, were obtained from Shizuoka Laboratory Animal Center (Hamamatsu, Japan). Proteinase-free chondroitin ABC lyase (EC 4.2.2.4) from *Proteus vulgaris*, hyaluronate lyase (EC 4.2.2.1) from *Streptomyces hyalurolyticus* and authentic glycosaminoglycans, hyaluronic acid from human umbilical cord, chondroitin sulphate A from whale cartilage, chondroitin sulphate B from pig skin, chondroitin sulphate C from shark cartilage and heparan sulphate from bovine kidney, were obtained from Seikagaku Corporation (Tokyo, Japan). Heparan-sulphate lyases I (EC 4.2.2.8) and II (EC 4.2.2.8) purified from *Flavobacterium heparinum* and the authentic unsaturated disaccharides 2-acetamido-2-deoxy-4-*O*-(4-deoxy- α -L-threo-hex-enopyranosyluronic acid)-D-glucose (Δ DiHS-OS), 2-acetamido-2-deoxy-4-*O*-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose (Δ DiHS-6S), 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-D-glucose (Δ DiHS-NS), 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- α -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose (Δ DiHS-diS₁), 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-2-*O*-sulpho- α -L-threo-hex-4-enopyranosyluronic acid)-D-glucose (Δ -DiHS-diS₂) and 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-2-*O*-sulpho- α -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose (Δ DiHS-triS) were prepared as described previously [26]. Collagenase from *Clostridium histolyticum* was from Worthington (Freehold, NJ, U.S.A.). The M_r standards used in gel filtration were pullulans, α -glucans (gifts from Hayashibara Biochemical Laboratories, Okayama, Japan), which were established as a series of M_r standards [27,28] and used in the present study for confirming the linearity of the calibration curve

as described previously [29], the three species of heparan sulphate proteoglycans isolated from cultured erythroleukemia cells [29], and the three species of chondroitin 6-sulphate proteoglycans from rabbit bone marrow whose M_r values were determined by sedimentation-equilibrium centrifugation [30]. Sepharose CL-4B and DEAE-Sephacel were purchased from Pharmacia LKB Biotechnology (Tokyo, Japan). Bio-Gel A-5 m was from Nippon Bio-Rad Laboratory (Tokyo, Japan). The proteinase inhibitors used were *N*-ethylmaleimide from Nacalai Tesque (Kyoto, Japan), phenylmethanesulphonyl fluoride from Sigma (St. Louis, MO, U.S.A.), pepstatin A from the Protein Research Foundation (Osaka, Japan) and Trasylol from Yoshitomi Pharmaceutical Co. (Osaka, Japan). Antiserum raised in rabbits against the BM-1 proteoglycan [31] purified from the Engelbreth-Holm-Swarm (EHS) tumour, was a gift from Dr John R. Hassell, University of Pittsburgh, Pittsburgh, PA, U.S.A. The antiserum was purified by passage through a column of laminin-linked Sepharose CL-4B and then by adsorption to a column of BM-1 proteoglycan-linked Sepharose CL-4B, and shown to react with its core protein [31,32]. Horseradish-peroxidase-conjugated swine antiserum against rabbit immunoglobulins and normal swine serum were obtained from DAKO (Copenhagen, Denmark).

Cell culture and formation of primary tumours

The Lewis-lung-carcinoma-cell clones P29, LM12-3 and LM60-D6, with low, medium and high metastatic potentials respectively, were established previously on the basis of their spontaneous metastatic potentials [13,24]. The cloned cell lines were routinely cultured in Dulbecco's modified Eagle's medium (GIBCO, New York, NY) containing 10% (v/v) foetal-bovine serum (GIBCO), penicillin (100 units/ml) and streptomycin (100 μ g/ml), at 37 °C in a humidified atmosphere of 5% CO₂ in air. Cells of the three clones were significantly different in shape and doubling time *in vitro*; P29 cells grew round in shape and had a doubling time of 10 h, LM12-3 cells were spindle-shaped and had a doubling time of 19 h, and LM60-D6 cells were more polygonal, with a doubling time of 18.5 h. Cells from the different clones also reached different saturation densities [13]. Cultured cells were detached from dishes by treatment with 2 mM-EDTA in Ca²⁺ and Mg²⁺-free phosphate-buffered saline (0.137 M-NaCl/2.7 mM-KCl/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄, pH 7.4) for 10 min, and subcultured twice a week.

To form primary tumours, single cell suspensions (2×10^5 cells/0.2 ml of Hanks' balanced salt solution/animal) were injected subcutaneously into the right abdominal flank of mice. After 1 month the animals were killed and the subcutaneous primary tumours were excised. Necrotic parts of tumours were carefully removed under a dissecting microscope. The tumour tissues were frozen at –80 °C until use. The growth of tumours formed *in vivo* was monitored by caliper measurement as described previously [24]. The sizes of the primary subcutaneous tumours formed by the low and the two more highly metastatic clones reached 3.0 cm in maximal diameters at 25 and 30 days respectively after implantation.

Immunohistochemistry

Immunohistochemistry of the primary tumours formed by the three clones using the anti-(BM-1 proteoglycan) antiserum was performed as described previously [13].

Quantitative analysis of glycosaminoglycans in tumour tissues

For quantitative analysis of glycosaminoglycans in tumour tissues, proteins and nucleic acids were removed as follows. Tissues (5–8 g wet weight) were defatted by successive treatments with acetone, diethyl ether, chloroform/methanol (2:1, v/v) and acetone, twice each at –20 °C for 2 h, dried and weighed. The

Fig. 1. Immunohistochemical staining, with anti-(BM-1 proteoglycan) antibody, of primary tumour tissues

The tumours were formed by subcutaneous injection of cell suspensions of the P29 (a), LM12-3 (b) and LM60-D6 (c) clones, and of the original (3LL) tumour (d) from which the three clones were derived, into the right abdominal flank of mice. Before staining with anti-(BM-1 proteoglycan) antibody, specimens were treated with bacterial collagenase. Closed and open arrowheads show tumour basement membrane and endothelial basement membrane of tumour blood vessels respectively. The bar represents 100 μm .

defatted samples were suspended in 2 mM- CaCl_2 /0.1 M-Tris/HCl, pH 8.0, boiled for 5 min and digested with Pronase (1:25, dry w/w) at 50 $^\circ\text{C}$ for 72 h under toluene with the further addition of half the amount of enzyme at 24 h intervals. The digests were dialysed against water and freeze-dried. The resulting residue was dissolved in 0.3 M-NaOH/1 M- NaBH_4 , left at room temperature overnight, neutralized with acetic acid and dialysed against water. The dialysed sample was adjusted to 5% (w/v) trichloroacetic acid and left overnight on ice. The precipitate, containing nucleic acids and peptides, was removed by centrifugation and discarded. The supernatant, in which more than 90% of total hexuronate-containing material was recovered, was collected and dialysed against water. The contents and species of glycosaminoglycans including hyaluronic acid, chondroitin sulphate and heparan sulphate in the samples were determined as described previously [33]. Briefly, the samples were digested sequentially with hyaluronate lyase [34] and chondroitin ABC lyase [35] and then treated with HNO_2 [36]. The sample obtained after each treatment was analysed on a column (1 cm $\times$ 32 cm) of Sephadex G-50, which was equilibrated and developed with 0.5 M-NaCl/50 mM-Tris/HCl, pH 7.3.

Extraction and isolation of heparan sulphate proteoglycans

The frozen tumours were cut into small pieces and defatted by successive treatments as described above, but all procedures were carried out in the presence of 10 mM-EDTA, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride, 0.036 mM-pepstatin A and Trasylol (5×10^4 units/l) as proteinase inhibitors. The acetone-dried powder (2.5–2.9 g) was weighed exactly, suspended in 220 ml of 4 M-guanidinium chloride/0.5% (v/v) Triton X-100/50 mM-Tris/HCl, pH 7.3, containing the same proteinase inhibitors, stirred on ice for 24 h and centrifuged at 140000 g for 40 min. The residue was resuspended in 80 ml of the same extraction buffer, stirred for 12 h, and then centrifuged. The final residue (guanidinium chloride-insoluble fraction) was suspended in 50 ml of water and stored at -80°C until use. The combined supernatant was dialysed on ice seven times against 3 litre volumes of 7 M-urea/0.5% (v/v) Triton X-100/20 mM-NaCl/20 mM-Tris/HCl, pH 7.3, containing 10 mM-EDTA and Trasylol (5×10^4 units/litre). The insoluble material appearing

during this dialysis was collected by centrifugation at 140000 g for 40 min, washed with 50 ml of dialysing buffer and dissolved in 50 ml of extraction buffer (urea-insoluble fraction). The supernatants were combined (urea-soluble fraction) and applied to a column of DEAE-Sephacel. The column was washed with 20 mM-NaCl in urea solution and eluted stepwise with 0.1 M-NaCl and then with 1 M-NaCl in urea solution. The 1 M-NaCl fraction, in which almost all of the hexuronate-containing macromolecules were eluted, was dialysed against water on ice and freeze-dried. Freeze-dried samples containing 540–640 μg of hexuronic acid were suspended in 5 ml of 0.1 M-sodium acetate buffer, pH 6.0, containing 10 mM-EDTA, 10 mM-*N*-ethylmaleimide, 1 mM-phenylmethanesulphonyl fluoride and 0.036 mM-pepstatin A. The samples were then incubated with 1 unit of proteinase-free chondroitin ABC lyase at 37 $^\circ\text{C}$ for 40 min to degrade hyaluronic acid and chondroitin sulphate. Under the conditions used, the rate of depolymerization of hyaluronic acid was about two thirds of that of chondroitin sulphate. The degraded materials were removed by dialysis against water on ice and heparan sulphate proteoglycans were freeze-dried.

Partial purification of heparan sulphate proteoglycans

The heparan sulphate proteoglycans obtained were dissolved in 3 ml of 4 M-guanidinium chloride/0.5% (w/v) CHAPS/50 mM-Tris/HCl, pH 7.3, and applied to a column of Sepharose CL-4B and eluted with the same buffered solution. The fractions containing heparan sulphate proteoglycans were pooled and concentrated. Solid CsCl (0.35 g/ml) was added to give an initial density of 1.35 g/ml, and a density gradient was formed by centrifugation at 170000 g for 72 h at 5 $^\circ\text{C}$. Aliquots of heparan sulphate proteoglycans (equivalent to 3.0 μg of hexuronate) fractionated by CsCl -density-gradient centrifugation were dissolved in 50 μl of 50 mM-Tris/HCl, pH 7.3, containing 2% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 0.04% (w/v) Bromophenol Blue, incubated for 2 h at 37 $^\circ\text{C}$, and then subjected to SDS/PAGE in a 5% running gel [37]. The proteoglycans were transferred to a nitrocellulose membrane [38] and their activities with the anti-(BM-1 proteoglycan) antibody were examined as described previously.

Chromatography

Column chromatography of samples on gels and DEAE-Sephacel was performed at 4 °C unless otherwise indicated. H.p.l.c. for analysis of unsaturated disaccharides was performed by using a Hitachi model 638-30 instrument with a column (2.6 mm × 250 mm) of chemically modified silica gel LiChrosorb NH₂ (Merck) as described previously [26].

Other methods

Hexuronate was measured by the method of Bitter & Muir [39] with glucuronolactone as a standard. The protein content was determined by the method of Lowry *et al.* [40], with BSA as a standard. Two-dimensional electrophoresis of glycosaminoglycan samples was carried out on a cellulose acetate membrane as described previously [41].

RESULTS

Metastatic potentials of the three clones

As the metastatic potential of cloned tumour cells often changes during their passage and storage, we firstly confirmed lung-colonizing abilities and spontaneous metastatic potentials of the three clones before experiments. The three clones showed significant differences in incidences of metastases and numbers of lung metastatic nodules (results not shown), the order of their metastatic abilities being LM60-D6 > LM12-3 > P29, as described previously [13], indicating that the metastatic potentials of the three clones had been well maintained.

Immunohistochemical staining of basement-membrane proteoglycan in primary subcutaneous tumours formed from the three clones

To examine the location of basement-membrane proteoglycan in the individual tumours, we stained primary tumour tissues with anti-(BM-1 proteoglycan) antiserum, which reacts with the core protein of basement-membrane proteoglycan. Extracellular compartments between tumour cells in the three differently metastatic tumours were positively stained with the antibody, but in different patterns (Fig. 1). In the P29 tumour (Fig. 1a), these structures appeared to be punctate or fragmented, whereas in the LM12-3 (Fig. 1b) and LM60-D6 (Fig. 1c) tumours, they were continuous, representing a basement-membrane nature. To clarify these differences, morphometrical analysis was performed using the specimens stained with the antibody. The results showed that the frequency of the more continuous basement membranes and the extent of their continuity were significantly greater in the more highly metastatic tumours, as shown in a previous study with anti-laminin and anti-(type IV collagen) antibodies [13]. The antibody used was also reactive with the vascular basement membrane, indicating that it reacts with the core protein(s) of the basement-membrane proteoglycan(s) produced by vascular endothelial cells. On the basis of the morphometrical results, the vascular basement membrane in each of the three individual tumours accounted for 11–15% of the total length of the antibody-reactive basement membrane (results not shown). These results were very similar to those obtained in the previous study using anti-laminin and anti-(type IV collagen) antibodies [13], and indicated that basement-membrane proteoglycan contributed to the construction of the structures in a manner characteristic of the individual tumours.

The staining pattern of the original tumour (3LL) (Fig. 1d) very much resembled those of the LM60-D6 tumour, suggesting that the highly metastatic cells are the most predominant variant in the original 3LL tumour. This is consistent with the fact that the 3LL tumour is malignant.

Quantitative analysis of glycosaminoglycans in tumours with different metastatic potentials

The molecular species and content of various glycosaminoglycans contained in the primary tumours formed by the three clones were determined as described in the Experimental section. The glycosaminoglycans comprised hyaluronic acid, chondroitin sulphate and heparan sulphate: the content of hyaluronic acid in the P29, LM12-3 and LM60-D6 tumours was found to be 144, 29 and 11 µg of hexuronate/g dry weight of tissue respectively; the corresponding values for chondroitin sulphate were 59, 117 and

Fig. 2. Gel chromatography on Sepharose CL-4B of heparan sulphate proteoglycans

Heparan sulphate proteoglycans of the P29 (a), LM12-3 (b) and LM60-D6 (c) tumours were isolated from the proteoglycans fractions from DEAE-Sephacel chromatography, which were treated with proteinase-free chondroitin ABC lyase at pH 6.0 in the presence of proteinase inhibitors to remove hyaluronic acid and chondroitin sulphates. The resulting samples were applied to a column (1.6 cm × 95 cm) of Sepharose CL-4B and eluted with 4 M-guanidinium chloride solution at a flow rate of 6 ml/h. Fractions (2.5 ml each) were collected, and hexuronate content (O) and A₂₈₀ (●) were monitored. The fractions named PGI, PGII and PGIII indicated by bars were pooled for further purification. Thick arrows show the void volume and the total volume of the column. Large thin arrows numbered 1, 2, 3 and 4 show the elution positions of pullulans with *M_r* values of 380 000, 186 000, 100 200 and 48 000 respectively and small thin arrows with letters a, b and c show those of heparan sulphate proteoglycans with *M_r* values of 360 000, 320 000 and 280 000, respectively.

Fig. 3. CsCl-density-gradient centrifugation and immunoblotting with anti-(BM-1 proteoglycan) antibody after SDS/PAGE of heparan sulphate proteoglycans of the three tumours

The PGII fractions derived from the P29 (a), LM12-3 (b) and LM60-D6 (c) tumours were adjusted to an initial density of 1.35 g/ml with solid CsCl and centrifuged at 170000 g for 72 h at 5 °C. Fractions (0.7 ml each) were collected. The hexuronate content (○), absorbance at 280 nm (●) and density (●) were monitored. The fractions PGII-B and PGII-M indicated by bars were pooled and small aliquots (3.0 μ g of hexuronate) were subjected to SDS/PAGE, followed by immunoblotting with anti-(BM-1 proteoglycan) antibody (insets). Arrowheads with letters 1, 2, 3, 4 and 5 show the positions of the marker proteins (thyroglobulin, ferritin, catalase, lactate dehydrogenase and BSA), with M_r values of 669000, 440000, 232000, 140000 and 67000 respectively.

99 μ g of hexuronate; and of heparan sulphate, 125, 155 and 158 μ g of hexuronate. An extremely high content of hyaluronic acid in the P29 tumour was apparently attributable to its production by fibroblast-like cells which were detectable immunohistochemically and ultrastructurally only in the P29 tumour, because cultured cells of the three clones did not synthesize hyaluronic acid and only the P29 tumour has a high potential to induce a stromal response in the surrounding connective tissue of host animals (N. Nakanishi, K. Oguri, K. Takenaga, S. Hosoda & M. Okayama, unpublished work). On the other hand, the contents of both chondroitin sulphate and heparan sulphate tended to be increased in the more highly metastatic tumours.

Extraction and isolation of heparan sulphate proteoglycans

For isolation of the heparan sulphates as proteoglycans, the three defatted tumours were extracted with 4 M-guanidinium chloride containing proteinase inhibitors, followed by extensive dialysis against 7 M-urea solution. The guanidinium chloride- and urea-insoluble fractions containing negligible amounts of hexuronate were not studied further. On DEAE-Sephacel ion-exchange chromatography of the urea-soluble fractions, 85–90% of the hexuronate-containing macromolecules applied were co-eluted with about 8% of the total protein with 1 M-NaCl (results not shown). Thus a large portion of the total protein was separated from this fraction. For the isolation of heparan sulphate proteoglycans, the 1 M-NaCl fraction was digested with proteinase-free chondroitin ABC lyase at low pH (pH 6.0) to degrade both hyaluronic acid and chondroitin sulphate. The digestion was carried out in the presence of proteinase inhibitors to prevent degradation by endogenous proteinases which could still be contaminating the 1 M-NaCl fraction. Under the reaction conditions used, hyaluronic acid was depolymerized at two-

thirds the rate of chondroitin sulphate. The digest was dialysed against water to remove the degraded materials and freeze-dried.

Characterization of heparan sulphate proteoglycans

The freeze-dried heparan sulphate proteoglycan samples were dissolved in 4 M-guanidinium chloride/0.5% CHAPS/50 mM-Tris/HCl, pH 7.3, and applied to a column of Sepharose CL-4B (Fig. 2). Heparan sulphate proteoglycans of all three tumours were eluted as three peaks: PGI in the void volume, PGII with a partition coefficient (K_D) of 0.13–0.55, and PGIII at a K_D of 0.66–0.90. From the elution positions of standard proteoglycans and pullulans, the M_r values of heparan sulphate proteoglycans PGII and PGIII were estimated to be 820000–130000 and less than 80000 respectively. The relative amounts of hexuronate eluted in the PGI, PGII and PGIII fractions accounted for 10, 57 and 33% respectively of the P29 tumour-derived sample; 8, 41 and 51% of the LM12-3 tumour-derived one; and 1, 33 and 64% of the LM60-D6 tumour-derived one. This result demonstrated that the pool size of the PGIII component in tissues increases significantly (41, 70 and 101 μ g of hexuronate/g dry weight of tissue of the P29, LM12-3 and LM60-D6 tumours respectively) with an increase in metastatic potential. The minor component, PGI, excluded from the column was not examined further in this study.

On CsCl-density-gradient centrifugation in 4 M-guanidinium chloride solution containing 0.5% CHAPS, proteoglycan in the PGII fraction from the three tumours was further separated into two fractions with different densities (Fig. 3): a bottom fraction (PGII-B) with a density of more than 1.37 g/ml, and a middle fraction (PGII-M) with a buoyant density of less than 1.34 g/ml. Because the results obtained immunohistochemically showed that the anti-BM-1 proteoglycan reacted with basement-membrane structures characteristic of the individual tumours, the

Fig. 4. Gel chromatography on Bio-Gel A-5m and two-dimensional electrophoresis on a cellulose acetate membrane of heparan sulphates released from the PGII-M proteoglycans of the P29 and LM60-D6 tumours

Heparan sulphate released by alkali/borohydride treatment of the PGII-M proteoglycan from the P29 (a) and LM60-D6 (b) tumours was applied to a column (1 cm × 113 cm) of Bio-Gel A-5m and eluted with 1 M-NaCl/50 mM-Tris/HCl, pH 7.3, at a flow rate of 4 ml/h. Fractions (1.2 ml each) were collected and their hexuronate content was monitored. Small arrows show the void volume and the total volume of the column. Arrows with letters a, b and c show the elution positions of chondroitin 6-sulphate proteoglycans with M_r values of 46 000, 16 000 and 8 300 respectively. The fractions indicated by bars were pooled for further study. Small portions (1 µg of hexuronate) of the samples were subjected to two-dimensional electrophoresis on a cellulose acetate membrane in 0.1 M-pyridine/0.47 M-formic acid, pH 3.0, for the first dimension ('1st') and 0.1 M-barium acetate, pH 8.0, for the second dimension ('2nd'), with the following authentic glycosaminoglycans: HA, hyaluronic acid; CS-A, chondroitin sulphate A; CS-C, chondroitin sulphate C; DS, chondroitin sulphate B; and HS, heparan sulphate as external references. The glycosaminoglycans on the membrane were stained with Alcian blue.

reactivity of these two large proteoglycans with the antibody was examined by immunoblotting (an amount equivalent to 3.0 µg of hexuronate for each) after SDS/PAGE (insets in Fig. 3). When the gels were stained with Toluidine Blue, both proteoglycans of the individual tumours showed similar electrophoretic patterns (results not shown). However, on staining with the antibody, only the heparan sulphate proteoglycan of the PGII-M fraction reacted with the antibody; that of the PGII-B fraction did not. These results indicated that all three tumour tissues contain at least two large molecular species of heparan sulphate proteoglycan with different immunogenicities, and that the heparan sulphate proteoglycan with low density was apparently involved in the construction of basement-membrane structures characteristic of the individual tumours. Therefore we concluded that the PGII-M proteoglycan was basement-membrane proteo-

Table 1. Distribution of the two large heparan sulphate proteoglycans in PGII-B and PGII-M fractions separated by CsCl-density-gradient centrifugation of the PGII proteoglycan fractions

Tumour	Hexuronate (µg/g dry weight of tumour)	
	PGII-B	PGII-M
P29	35	36
LM12-3	30	34
LM60-D6	15	37

Fig. 5. H.p.l.c. analyses of digests of the heparan sulphates released from the PGII-M proteoglycans of the three tumours by a combination of heparan-sulphate lyases I and II

The heparan sulphates of the PGII-M proteoglycans from the P29 (a), LM12-3 (b) and LM60-D6 (c) tumours were digested with a combination of heparan-sulphate lyases I and II, and the unsaturated disaccharides produced were analysed by h.p.l.c. The retention times of the individual unsaturated disaccharides were determined by using authentic unsaturated disaccharides (d).

glycan. The PGII-M proteoglycan contents of the three tumours were very similar, whereas those of PGII-B proteoglycan decreased with increasing metastaticity of the tumours (Table 1). The PGII-B proteoglycan was not studied further. The small proteoglycan in the PGIII fraction was also examined for reactivity with anti-(BM-1 proteoglycan) antibody as described above, but the results indicated that the proteoglycan from all three tumours did not react with the antibody at all (results not shown).

For the preparation of heparan sulphates from the PGII-M

proteoglycan of the three tumours, the proteoglycan samples were treated with 0.3 M-NaOH in the presence of 1 M-NaBH₄ overnight, followed by treatments with Pronase and cold trichloroacetic acid. Heparan sulphates collected in the trichloroacetic acid-soluble fraction were analysed by gel chromatography on Bio-Gel A-5m (Fig. 4). Both the P29 and LM60-D6 tumour-derived heparan sulphates were eluted from the column as a sharp symmetrical peak at a position corresponding to a K_D value of 0.48. The elution profile and position of the LM12-3 tumour-derived heparan sulphate were very similar (results not shown). On the basis of the calibration curve, the M_r values of the heparan sulphates of the three tumours were all estimated to be about 36000. On two-dimensional electrophoresis, the individual heparan sulphates migrated as single compact spots and nearly coincided in position with bovine kidney heparan sulphate used as a reference (Fig. 4, right panels), indicating that these heparan sulphates had a very homogenous charge density. However, the LM60-D6 tumour-derived heparan sulphate migrated significantly faster in the first dimension than that from the P29 tumour, suggesting that the former was more negatively charged than the latter. This result was consistent with findings from h.p.l.c. analysis of their disaccharide units, as shown below.

The heparan sulphate samples obtained from the PGII-M proteoglycans were digested with a combination of heparan-sulphate lyases I and II. As the degree of degradation by this digestion of the heparan sulphates from the three tumours was examined by gel-permeation h.p.l.c. on a TSK-gel column, the heparan sulphates were almost completely depolymerized to unsaturated disaccharides (results not shown). The digests were then subjected to h.p.l.c. on an amine-bound silica column with a linear gradient of 16 mM- to 800 mM-NaH₂PO₄ (Fig. 5). More than 92% of the disaccharides generated from the three samples were eluted with the same retention times as those of the six authentic disaccharides. The remaining minor unidentified compounds, probably having unsaturated hexuronic acid at their non-reducing ends, were eluted as six peaks (X1-X6 in Fig. 5). The relative contents of the six identified disaccharides of the individual samples are summarized in Table 2. A non-sulphated unsaturated disaccharide, Δ DiHS-OS, was the major component (45–56%) of total disaccharides produced from all three samples, and the *N*-sulphated disaccharide, Δ DiHS-NS, was the second most prominent (22–25%). The disaccharides having an *N*-

Table 2. Compositions of unsaturated disaccharides produced by digestion of heparan sulphates derived from the PGII-M proteoglycan of the differently metastatic tumours with a combination of heparan-sulphate lyases I and II

Tumour... Unsaturated disaccharide	Composition					
	P29		LM12-3		LM60-D6	
	(nmol)	(%)	(nmol)	(%)	(nmol)	(%)
Applied	44.3	100	42.2	100	42.6	100
Recovered						
Δ DiHS-OS	23.2	52.4	18.9	44.8	17.9	42.0
Δ DiHS-6S	0.7	1.5	1.2	2.8	1.7	4.0
Δ DiHS-NS	10.1	22.8	8.4	19.9	9.5	22.3
Δ DiHS-diS ₁	0.4	1.0	2.3	5.5	2.7	6.3
Δ DiHS-diS ₂	4.3	9.8	3.6	8.5	3.6	8.5
Δ DiHS-triS	2.0	4.4	4.2	10.0	4.7	11.0
Sum...	40.7	91.9	38.6	91.5	40.1	94.1

sulphate residue, which is specific to heparin-like molecules, accounted for 42–52% of the total disaccharides. It is noteworthy that there is a significant decrease in the amount of the non-sulphated repeating disaccharide in association with an increase in the metastatic potential of the tumours. The increased degree of sulphation of the heparan sulphate of the more highly metastatic tumours was due to an increased content of sulphated repeating disaccharides having 6-*O*-sulphated glucosamine residues (shaded areas in Fig. 6).

Finally, we characterized the small proteoglycan, PGIII, which did not react with the anti-(BM-1 proteoglycan) antiserum. When heparan sulphates released by β -elimination of the PGIII proteoglycan were chromatographed on Sepharose CL-4B, the elution positions were not significantly changed as compared with those of the proteoglycans before β -elimination treatment (results not shown), indicating that the PGIII proteoglycan of all the tumour tissues had only small peptides, if any. On chromatography on Bio-Gel A-5m (Fig. 7), the heparan sulphate from the P29 tumour-derived PGIII proteoglycan was eluted as two peaks of hexuronate, which were not separated completely, at positions with K_D values of 0.55 and 0.70. From the calibration curve, the M_r of the larger glycosaminoglycan was estimated to

Fig. 6. Schematic illustration of an increase of repeating disaccharide units with 6-*O*-sulphated glucosamine residues in the heparan sulphate of the basement-membrane proteoglycan PGII-M associated with an increase in the metastatic potential of the tumours

The illustration represents the relative values calculated on the assumption that all the heparan sulphates are completely converted into the identified six unsaturated disaccharides by digestion with heparan-sulphate lyases I and II. However, the digests of the P29-, LM12-3- and LM60-D6-tumour-derived samples also contained the unidentified unsaturated oligosaccharides shown as X1-X6 in Fig. 5, which accounted in total for 8.1, 8.5 and 5.4% of the total unsaturated oligosaccharides respectively (see Table 2).

Fig. 7. Gel chromatography on Bio-Gel A-5m of heparan sulphates released from the PGIII proteoglycans of the P29 and LM60-D6 tumours

Heparan sulphates of the PGIII proteoglycan from the P29 (a) and LM60-D6 (b) tumours were chromatographed under the same conditions as those described in Fig. 4. Small arrows show the void volume and the total volume of the column. Large arrows with letters a, b and c show elution positions of chondroitin 6-sulphate proteoglycans with M_r values of 46000, 16000 and 8300 respectively.

Table 3. Compositions of unsaturated disaccharides produced by digestion of heparan sulphates derived from the PGIII proteoglycan of the differently metastatic tumours with a combination of heparan-sulphate lyases I and II

Tumour ...	Composition			
	P29		LM60-D6	
Unsaturated disaccharide	(nmol)	(%)	(nmol)	(%)
Applied	43.8	100	44.7	100
Recovered				
ΔDiHS-OS	23.1	52.7	17.4	38.9
ΔDiHS-6S	0.4	0.9	1.5	3.3
ΔDiHS-NS	8.7	19.9	13.8	30.0
ΔDiHS-diS ₁	0.8	1.8	2.0	4.5
ΔDiHS-diS ₂	5.1	11.6	4.2	9.4
ΔDiHS-triS	1.9	4.3	3.4	7.6
Sum ...	40.0	91.2	42.3	94.6

be about 26000 and that of the smaller one to be 11000. The LM60-D6 tumour-derived heparan sulphate was also composed of these two components, but their relative amounts were significantly different. These two fractions were pooled and subjected to disaccharide analysis on h.p.l.c. after digestion with heparan sulphate lyases I and II as described above. The results

obtained by analysis on TSK-gel h.p.l.c. indicated that both tumour-derived heparan sulphates were almost completely depolymerized (results not shown). H.p.l.c. analyses on an amine-bound silica column demonstrated that more than 91 % of the unsaturated disaccharides had the same retention times as the six authentic disaccharides. The additional six minor unidentified compounds (X1-X6), which had the same retention times as those generated from the heparan sulphates of the PGII-M proteoglycan, were also detected. The relative amounts of the six identified disaccharides generated from these PGIII-derived heparan sulphates in the two tumours were very similar to the relative amount of those derived from the PGII-M proteoglycan obtained from the corresponding tumours (Table 3).

DISCUSSION

Our previous study showed that an '*in vivo*' selection of clones with different metastatic potentials from Lewis lung carcinoma (3LL) resulted in the selection of clones with different abilities to form basement-membrane-like structures *in vivo*, that is, more highly metastatic clones possessed the capacity to form more highly organized tumour basement membrane *in vivo*, as judged on the basis of continuity and integrity [13]. Here we have demonstrated immunohistochemically that heparan sulphate proteoglycan, in addition to laminin and type IV collagen [13], was also involved in organization of the basement-membrane structures characteristic of the individual tumours.

Of three molecular species of heparan sulphate proteoglycans present in all the three tumours, two large-hydrodynamic-size species with low (PGII-M) and high (PGII-B) densities, and a small-hydrodynamic-size component (PGIII), only the PGII-M proteoglycan reacted with the anti-(BM-1 proteoglycan) antibody and was so identified to be involved in formation of the basement-membrane-like structures observed in the individual tissues. The contents of this proteoglycan in the three tumours were very similar (34-37 μg of hexuronate/g dry weight). On the other hand, the content of the PGII-B proteoglycan was significantly higher in the low-metastatic tumour (35 μg of hexuronate/g dry weight) than in the most highly metastatic tumour (about 15 μg /g dry weight). Our preliminary results showed that the three clones synthesized *in vitro* a heparan sulphate proteoglycan with hydrophobic properties that was separable from the anti-(BM-1 proteoglycan) antibody-reactive heparan sulphate proteoglycan and which had a high molecular density, identifiable as the PGII-B proteoglycan described here (N. Itano, K. Oguri, H. Nakanishi & M. Okayama, unpublished work). These results indicate that Lewis-lung-carcinoma cells synthesize at least two classes of heparan sulphate proteoglycans, differing in immunogenicity, hydrophobicity and molecular density. On the basis of these results, together with other previous observations that several epithelial cell types synthesize both basement-membrane and cell-membrane heparan sulphate proteoglycans [42,43], it appears reasonable to conclude that, in addition to the basement-membrane proteoglycan (PGII-M), Lewis-lung-carcinoma cells synthesize the PGII-B proteoglycan as a cell-membrane component.

The present study clearly demonstrated that there was a close correlation between the degree of sulphation of heparan sulphate of the basement-membrane proteoglycan and the degree of organization of the tumour basement membrane of the individual tumours; the average molar ratios of sulphate residues to repeating disaccharides of the heparan sulphates from the P29, LM12-3 and LM60-D6 tumours were 0.59, 0.81 and 0.89 respectively. On the other hand, the laminin and type IV collagen synthesized by the three clones *in vitro* did not show any recognizable differences in their molecular characteristics [44].

Therefore, the reduced level of continuity and integrity of the tumour basement membrane in the lower metastatic tumour could be, at least in part, attributable to the reduced extent of sulphation of heparan sulphate chains of the basement-membrane proteoglycan. This similar correlation has been demonstrated using a mouse mammary epithelial cell line and its spontaneously transformed counterpart [17,18]; the transformed cells showed a poor formation of basement membrane both *in vivo* and *in vitro* and synthesized basement-membrane proteoglycan having heparan sulphate chains with a reduced extent of *N*- and *O*-sulphations as compared with normal cells. In addition, there are many reports indicating that a decreased degree of sulphation of heparan sulphates is associated with oncogenic transformation of cells, and it is generally considered to be responsible for the impaired ability of the glycosaminoglycans and the cells to interact with other extracellular-matrix components [19,20]. In these previous papers the relationships between normal cells and transformed cells have been expanded to tumour progression such as invasion or metastasis. Thus, in the case of tumour basement membranes, more malignant cells have been proposed to have a reduced ability to form a basement membrane, favouring their dissemination from their original sites [17,18]. However, in contrast with the previous hypothesis proposed by these authors on tumour progression [19,20], the more highly metastatic cells, at least in the Lewis-lung-carcinoma system used in the present study, formed the more highly organized basement membrane *in vivo*, which contained the proteoglycan having the more highly sulphated heparan sulphate chains. These results, together with others cited above, indicate that oncogenic transformation and tumour progression occurring after the transformation should be examined as a different problem. In any case, to the best of our knowledge, the present study is the first systematic examination of alterations of the isomeric structures of heparan sulphates of the basement-membrane proteoglycan during tumour progression.

In respect to the reduced degree of sulphation of heparan sulphate of the basement-membrane proteoglycan, it is notable that the relative content of 6-*O*-sulphated glucosamine residues increased in the more highly metastatic tumours (6.9, 18.3 and 21.3 % of the total disaccharides of the P29, LM12-3 and LM60-D6 tumour-derived glycosaminoglycans respectively). Winterbourne & Mora [45] reported that a specific reduction in 6-*O*-sulphation of glucosamine residues of heparan sulphate in the extracellular matrix occurred in association with the oncogenic transformation of mouse embryonic fibroblasts and was associated with a reduced affinity for a specific cell receptor on the cells [46]. These results cannot simply be compared with the present ones because of differences in the cell types and systems used. However, it is reasonable to consider that sulphation at position 6 of glucosamine residues in certain specific sites of heparan sulphate chains is probably crucial for the formation of functional domains that interact with other molecules. To clarify functional aspects of these heparan sulphates, much sequencing information is required.

The findings that the PGIII proteoglycan had only a small peptide, if any, and its heparan sulphates were smaller than those of the two larger proteoglycans indicate that it is generated by degradation of both glycosaminoglycan chains and a core protein(s). However, it remains to be resolved in the present study whether it is produced from the two large, PGII-M and PGII-B, proteoglycans or either of them. In any case, the noteworthy feature of the PGIII proteoglycan was its large pool size in all three tumours, the pool size being largest in the most highly metastatic tumour. The existence of such a large pool of degraded heparan sulphate proteoglycan has not been reported in normal [47,48] or benign tumour [49] tissues. It would,

therefore, be taken to be characteristic of malignant carcinoma. The increased pool size in the more highly metastatic tumour might be attributable to an increased degradation of heparan sulphate proteoglycan, as demonstrated in a mouse melanoma system [50,51] and other various malignant tumours [52–55].

The previous [13] and present studies have clearly demonstrated that tumour basement membranes, even the most highly organized one formed by the most highly metastatic clone, are distinctly different from normal epithelial basement membranes with respect to localization and continuity. The latter exist as thin continuous sheets separating epithelial tissues from connective tissues, and are never located between epithelial cells, whereas the tumour basement membranes in the present tumours run randomly between tumour cells and are very discontinuous even in the most highly metastatic tumour. Electron-microscopic observation in the previous study [13] showed that the tumour basement membranes ran parallel, and often fused with each other to become a single basement membrane. In the vicinity of tumour blood vessels, similar interactions between the tumour basement membrane and the vascular basement membrane took place, resulting in the close contact of tumour cells with the vascular basement membrane and also in their regular arrangement around blood vessels. These interactions were more obvious in the more highly metastatic tumours having the more highly organized basement membranes. This kind of basement membrane fusion, 'back-to-back' fusion of basement membranes of dual origin, has been demonstrated when murine mammary carcinoma cells invaded the lung parenchyma [12]. Similar interactions were also observed in the primary tumours of human gastric cancers with liver metastasis, but not in those without it [14]. In the tumour cells used in this study, the more highly metastatic cells exhibit greater attachment than the low metastatic cells to the subendothelial matrix, probably reflecting the above '*in vivo*' situation [13]. On the basis of the results described above, it is conceivable that the metastatic potentials of tumour cells of epithelial origin are closely related to their adhesiveness to the vascular basement membrane, which is mediated in part by the interaction of tumour basement membranes with the vascular basement membrane. However, the dynamic aspects of basement membranes as described above are largely unknown. Also, we have no evidence that the attachment of tumour cells to the vascular basement membrane results in intravasation of the tumour cells. However, it is very likely that the more frequent and regular alignment of tumour cells on the vascular basement membrane, mediated by the more highly organized tumour basement membrane, may increase the chance of intravasation of tumour cells, permitting their dissemination to distant organs.

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