Epidermal transformation leads to increased perlecan synthesis with heparin-binding-growth-factor affinity

Pairath TAPANADECHOPONE, Sarka TUMOVA¹, Xinnong JIANG and John R. COUCHMAN²

Department of Cell Biology, Cell Adhesion and Matrix Research Center, University of Alabama at Birmingham, 201C Volker Hall, 1670 University Blvd., Birmingham, Alabama 35294-0019, U.S.A.

Perlecan, a proteoglycan of basement membrane and extracellular matrices, has important roles in both normal biological and pathological processes. As a result of its ability to store and protect growth factors, perlecan may have crucial roles in tumour-cell growth and invasion. Since the biological functions of different types of glycosaminoglycan vary with cellular origin and structural modifications, we analysed the expression and biological functions of perlecan produced by a normal epidermal cell line (JB6) and its transformed counterpart (RT101). Expression of perlecan in tumorigenic cells was significantly increased in both mRNA and protein levels. JB6 perlecan was exclusively substituted with heparan sulphate, whereas that of RT101 contained some additional chondroitin sulphate. Detailed structural analysis of the heparan sulphate (HS) chains from

perlecan of both cell types revealed that their overall sulphation and chain length were similar (≈ 60 kDa), but the HS chains of tumour-cell-derived perlecan were less sulphated. This resulted from reduced 2-O- and 6-O-sulphation, but not N-sulphation, and an increase in the proportion of unsulphated disaccharides. Despite this, the heparan sulphate of RT101- and JB6-derived perlecan bound fibroblast growth factor-1, -2, -4 and -7 and heparin-binding epidermal growth factor with similar affinity. Therefore abundant tumour-derived perlecan may support the angiogenic responses seen *in vivo* and be a key player in tumorigenesis.

Key words: fibroblast growth factors, heparan sulphate, proteoglycan, sulphation.

INTRODUCTION

During the development of primary tumours or the establishment of metastatic foci, there is a continuous remodelling of the extracellular matrix, which results from interactions between tumour and host cells [1]. Many studies have shown that neoplastic transformation dramatically alters proteoglycan (PG) synthesis, both in tumour and surrounding tissues. These quantitative and qualitative alterations are believed to promote tumorigenic growth and invasion [1–3]. The alterations in PG structure that appear to be directly linked to transformation can occur by expression of different types of glycosaminoglycan (GAG) chains, or an alteration in degree of sulphation [4,5]. Previously it has been demonstrated that some tumours secrete increased amounts of chondroitin sulphate PG (CSPG) [6,7], while others produce heparan sulphate PG (HSPG) predominantly [4,8]. Differences in PG structure in tumour cells could be responsible for differences in their proliferation and invasion properties. Different types of GAG and various degrees of sulphation may affect growth, partly because of the different binding affinities of GAGs to various cytokines and growth factors [9].

Perlecan mRNA and protein depositions have been shown to be markedly increased in some tumour cells as compared with normal tissue [10,11]. This suggests that it may play a pivotal role in invasion and/or tumour angiogenesis. High levels of perlecan deposition are present in newly vascularized stroma of some

tumours, suggesting its role in angiogenesis [1,12]. In addition, blocking perlecan expression by stable transfection of human colon-carcinoma cells with perlecan antisense cDNA resulted in suppression of tumour growth, neovascularization, as well as cellular invasion [13]. Perlecan core protein has been shown to serve as a ligand for integrin-receptor binding [14] as well as fibroblast growth factor-7 (FGF-7; [13]), while the GAGs promote binding of many FGFs to their receptors, with a potent induction of mitogenic and angiogenic activities [15,16]. This evidence supports the notion that perlecan is directly involved in promoting the growth and invasion of tumour cells. However, there is also a recent report indicating that perlecan may inhibit the growth and invasiveness of fibrosarcoma cells through an FGF-2-independent pathway [17]. This raises the possibility that perlecan may prevent the infiltration of mesenchymal neoplasm.

Perlecan is predominantly expressed as an HSPG, but in some circumstances it can be expressed as a chondroitin/dermatan sulphated or a hybrid molecule [18–20]. Furthermore, GAGs, HS in particular, can be composed of disaccharide subunits with specific sulphation patterns. These sequences contain templates that determine some biological functions of PG [21]. For example, during neural development, HS chain structures on perlecan-related HSPG change, resulting in altered affinities for FGF-1 and FGF-2, growth factors involved in the developmental regulation [22–24]. The structural and functional differences of perlecan in tumorigenic and non-tumorigenic cells have not been examined conclusively. We hypothesized that the GAGs of

Abbreviations used: GAG, glycosaminoglycan; CS, chondroitin sulphate; DS, dermatan sulphate; PG, proteoglycan; HS, heparan sulphate; FGF, fibroblast growth factor; RT-PCR, reverse transcriptase PCR; SAX, strong anion-exchange chromatography; HB-EGF, heparin-binding epidermal growth factor; rh, recombinant human; MMP, matrix metalloproteinase; AMV, avian myeloblastosis virus; *Tfl*, *Thermus flavus*; Δ UA-GlcNAc, unsaturated uronic acid-N-acetylglucosamine; Δ UA-GlcNSO₃, unsaturated uronic acid-N-sulphated glucosamine; Δ UA(2S)-GlcNS(6S), unsaturated uronic acid (2-sulphate)-N-sulphated glucosamine 6-sulphate; IdoUA(2S)-GlcNS, iduronic acid (2-sulphate)-N-sulphated glucosamine; GlcUA-GlcNAc($\pm$ 6S), glucuronic acid-N-acetylglucosamine ($\pm$ 6-sulphate).

¹ Present address: Department of Biosciences, University of Helsinki, 00014 Helsinki, Finland.

² Present address and address for correspondence: Imperial College School of Medicine, Sir Alexander Fleming Building, Kensington, London SW7 2AZ, U.K. (e-mail j.couchman@ic.ac.uk).

tumour-cell-derived perlecan are structurally distinct from those of the wild-type. These differences may affect the functional roles of perlecan in tumour growth and contribute to the process of tumour formation and progression. To test this hypothesis, we used JB6 Cl 41.5a cells, a mouse epidermal-cell model of tumour promotion. This cell line can be irreversibly transformed to an anchorage-independent and tumorigenic phenotype (RT101 cells) by treatment with the phorbol ester PMA ('TPA') [25,26]. Furthermore, when injected into immunocompromised rats, tumour growth and substantial angiogenic responses ensue (P. Tapanadechopone, X. Jiang and J. R. Couchman, unpublished work). This does not occur with the non-transformed JB6 line. We analysed the expression of perlecan extracted from both cell types and found that the expression of perlecan core protein and fine structure of the GAG chains from these cell types are different. However, there was no loss of high-affinity growth-factor binding, indicating a potential role for perlecan to promote the tumour-invasion process.

EXPERIMENTAL

Cell culture and metabolic labelling

JB6 Cl 41.5a cells, a mouse epidermal-cell line, and its transformed counterpart (RT101), were grown in Eagle's minimal essential medium supplemented with 5% (v/v) heat-inactivated foetal-bovine serum and 2 mM L-glutamine. Both cell lines were kindly provided by Dr Nancy H. Colburn (National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.) via Dr. Pi-Ling Chang and Dr. Charles Prince (University of Alabama at Birmingham). Mycoplasma testing of cells by DNA fluorochrome staining was conducted monthly. For radiolabelling, cells were cultured in the presence of 50 μ Ci/ml of inorganic [35 S]sulphate (NEN[®] Life Science, Boston, MA, U.S.A.) and/or 30 μ Ci/ml D-[3 H]glucosamine hydrochloride (American Radiolabeled Chemicals, St. Louis, MO, U.S.A.).

Ribonuclease protection assay

A fragment of 174 bp cDNA of mouse perlecan domain V was made by PCR using a domain IV/V cDNA as a template. The product encompassed residues 10266–10439 of perlecan [27]. The PCR product was subcloned into pBluescript SK (+), then linearized with restriction endonuclease *SaI*, which cleaves immediately downstream of the sequence. The linearized template DNA was subjected to phenol/chloroform extraction and ethanol precipitation. The DNA was then dissolved in RNase-free water.

The linearized DNA template was transcribed by T_7 RNA polymerase using MAXIscript[®] *in vitro* transcription kits (Ambion, Austin, TX, U.S.A.). The reaction was performed at 37 °C for 30 min–1 h in the presence of 10 mM each of ATP, CTP, GTP, 12.5 μ M of [α - 32 P]UTP and ribonuclease inhibitor. The DNA template was removed by incubating the reaction mixture with 2 units of RNase-free DNase I at 37 °C for 15 min. The RNA was precipitated by ammonium acetate/ethanol and resuspended in RNase-free water.

Total RNA was extracted from confluent (approx. 6×10^6 cells) JB6 and RT101 cells grown in T-75 flasks. Cells were lysed by addition of 3 ml of RNeasy[®] B (Leedo Medical Laboratories, Houston, TX, U.S.A.) and subjected to chloroform extraction. Cell debris was removed and high-molecular-mass nucleic acid was precipitated with propan-2-ol. The RNA pellets were dissolved in RNase-free water. For each sample, 5 μ l of 32 P-labelled antisense probe ($\approx 10^4$ – 10^5 c.p.m./ μ l) was mixed with an equal amount of total RNA from both JB6 and RT101

samples and incubated at 37 °C overnight for hybridization. The mixtures were subsequently added to ribonuclease digestion buffer containing 5 units of RNase A, 1000 units of RNase T1 and incubated at 37 °C for 30 min. To each reaction mixture was added 20 μ l of 10% (w/v) SDS, and 10 μ l of Proteinase K (20 mg/ml) and the mixture incubated for another 30 min. Hybridized RNAs were then extracted from the aqueous phase by precipitation with propan-2-ol. The RNA pellets were dissolved in RNA loading buffer, incubated at 90 °C for 3 min and subjected to electrophoresis on denaturing 5%-(w/v)-polyacrylamide gel. The gel was dried and subjected to autoradiography for quantification.

Reverse transcriptase PCR (RT-PCR) analysis of perlecan mRNA expression

cDNAs were synthesized from 1 μ g of each total RNA from both JB6 and RT101 cells using an Access RT-PCR system (Promega, Madison, WI, U.S.A.). Oligonucleotide primers for a C-terminal fragment of domain V perlecan (D FV; [27]) were used for amplification. The primers were 5'-GAGAATTCGACAGCAC-AGCCGAT-3' (forward) and 5'-GCGCGCGTCGACTACTC-GACACTGTC-3' (reverse). The reactions were performed using 1 μ M of each primer, 0.2 mM dNTP mix, 1 mM MgSO₄, 0.1 unit of avian-myeloblastosis-virus (AMV) RT, 0.1 unit of *Thermus flavus* (*Tfl*) DNA polymerase, and 1 μ g of each total RNA in AMV/*Tfl* reaction buffer. For first-strand cDNA synthesis, incubation was carried out at 48 °C for 45 min, followed by 94 °C for 2 min. For second-strand cDNA synthesis and PCR amplification, 40 cycles of 94 °C for 30 s, 60 °C for 1 min, 68 °C for 2 min were performed. A final extension of 68 °C for 7 min was performed at the end of 40 cycles. β -Actin internal standard (Ambion) was used as a control. The RT-PCR products were analysed on 1%-agarose gel. The perlecan primers were omitted for negative controls.

PG purification from conditioned medium of JB6 and RT101 cells

Conditioned media were centrifuged at 160 *g* for 15 min to remove debris and run through 3 cm $\times$ 1 cm DEAE-Sephacel (Pharmacia Biotech) columns equilibrated with 4 M urea, 0.2 μ M NaCl, 10 mM *N*-ethylmaleimide, 20 mM EDTA, 0.05 M Tris/HCl, pH 8.0, containing 0.2 mM PMSF and 0.1% Tween 20. The columns were washed with the above buffer, but at pH 4.0, and containing 50 mM sodium acetate instead of Tris/HCl, and were washed again with the same buffer but in the absence of urea. PGs were subsequently eluted with 4 M guanidinium chloride, pH 4.0, containing 50 mM sodium acetate and 0.1% Tween. Fractions (1 ml) were collected and UV absorbance at 280 nm was used to detect PGs. Corresponding fractions were pooled, and PGs were precipitated by adding 4 vol. of ice-cold ethanol and incubated at –20 °C for at least 3 h. The precipitated pellets were dried and resuspended in appropriate buffers.

Immunoprecipitation of perlecan from radiolabelled cultured cells

Protein A–Sepharose beads were added to radiolabelled conditioned media collected from both JB6 and RT101 cells and incubated for 1 h at room temperature to remove non-specific binding components. After bead sedimentation, supernatants were transferred to new tubes, and incubated with rabbit polyclonal antibody against perlecan core protein (EY#10, a gift from Dr. J. R. Hassell, Shriners Hospital for Children, Tampa, FL, U.S.A.), that was first bound to further batches of Protein A–Sepharose beads. After incubation, extensive washing with 0.1% Tween in Tris-buffered saline [50 mM Tris/HCl

(pH 7.5)/500 mM NaCl] was performed until a very low background radioactivity was detected in washes. Perlecan was eluted from the beads with 4 M guanidinium chloride and subjected to ethanol precipitation.

Gradient anion-exchange chromatography

Intact or chondroitinase ABC-treated [^{35}S]perlecan samples from JB6 or RT101 cells were applied to a 3 cm $\times$ 1 cm column of DEAE-Sephacel as described above, except the labelled perlecan was eluted with a gradient of 0.2–1.5 M NaCl/50 mM sodium acetate/10 mM *N*-ethylmaleimide, pH 4.0. Fractions (1 ml each) were collected and each analysed for conductivity and radiolabel content [28].

Gel electrophoresis and immunoblotting

Precipitated PG pellets from the previous step were dissolved in heparinase buffer (0.1 M sodium acetate/0.1 mM calcium acetate, pH 7.0). Aliquots of 15 μl , with or without digestion with 0.5–1 munits of heparinase III (heparitinase I, EC 4.2.2.8) and/or 1–2 munits of chondroitinase ABC (chondroitinase ABC lyase, EC 4.2.2.4, Seikagaku American Inc., Ijamsville, MD, U.S.A.) at 37 °C for 3–5 h were resolved by SDS/PAGE using a 3–15% (w/v)-polyacrylamide gradient, with subsequent transfer to nitrocellulose for immunoblotting as previously described [27]. Primary rat monoclonal antibody H5L5 recognizing domain III of mouse perlecan and a rabbit polyclonal antibody EY#10 [27] recognizing all perlecan core-protein domains were used. Alternately biotin-conjugated monoclonal antibodies recognizing chondroitin 'stubs' remaining after chondroitinase ABC were used at 2.5 $\mu\text{g}/\text{ml}$. These were antibody 1-B-5 (against unsaturated chondroitin disaccharide, 'ADi-0S'), 2-B-6 (against unsaturated chondroitin 4-sulphate disaccharide, 'ADi-4S') and 3-B-3 (against unsaturated chondroitin 6-sulphate disaccharide, 'ADi-6S') [19]. All three antibodies were from Seikagaku America. Alkaline phosphatase or horseradish peroxidase conjugates of goat anti-rat IgG or goat anti-rabbit IgG, diluted 1:3000 in the same buffers as the primary antibodies, were used as secondary antibodies for 1 h at room temperature. For the stub antibodies at 1:1500 dilution of horseradish peroxidase-conjugated streptavidin was used. Further extensive washing of membranes was followed by colour development (Bio-Rad) or ECL[®] (enhanced chemiluminescence) Western-blotting analysis system (Amersham Life Science, Piscataway, NJ, U.S.A.) according to the manufacturers' instructions.

Slot-blot analysis of perlecan

PGs were isolated from the conditioned media as described above. In this case, the monolayers were trypsinized and cell numbers were counted after media collection to normalize perlecan synthesis to cell number. Total PG concentrations were calculated by measuring the absorption at 280 and 260 nm:

$$[\text{Protein}] (\text{mg}/\text{ml}) = (1.5 \times A_{280}) - (0.75 \times A_{260})$$

Aliquots of total PG produced by the same number of cells, or in other experiments, the same concentration of total PG regardless of the cell number, were subjected to enzymic treatment with combined heparinase III and chondroitinase ABC to remove the GAGs. Two-fold serial dilutions of the samples were applied to nitrocellulose membrane (Bio-Rad Laboratories) assembled in Bio-Rad slot-blot apparatus. Membranes were blocked with 5% (w/v) dried milk in PBS for 30 min–1 h. Hybridoma supernatant H5L5 recognizing mouse perlecan domain III, diluted 1:8 in PBS containing 1% dried milk, 0.1% BSA and

0.1% Tween 20, was incubated with the membranes and processed as described above. The blots were developed by chemiluminescence detection and quantified by densitometer.

Perlecan GAG analysis

Purified perlecan immunoprecipitates were subjected to alkaline elimination as previously described [28]. [^{35}S]GAGs from both JB6 and RT101 perlecan were ethanol-precipitated then resuspended in heparinase or chondroitinase buffers [27]. For size analysis, released GAGs were analysed by HPLC chromatography on TSK 4000 (TosoHass G.m.b.H., Montgomeryville, PA, U.S.A.) equilibrated with 4 M guanidinium chloride/50 mM Tris/0.5% Triton X-100, pH 6.0, at a flow rate of 0.5 ml/min. The column was calibrated with heparin, HS and CS polysaccharides of known molecular mass. Aliquots (0.5 ml each) were taken for liquid-scintillation counting.

To verify the type of GAGs, either separate or combined heparinase I and III, or chondroitinase ABC were added and incubated at 37 °C to cleave HS and CS/dermatan sulphate (DS) GAGs respectively. After 6 h of incubation, additional enzyme aliquots were added and incubated overnight to ensure completion. Both combined heparinase- and chondroitinase-treated radiolabelled products were resolved on Superdex[®] Peptide HR 10/30 HPLC column (Amersham Pharmacia Biotech) equilibrated with 0.5 M pyridine acetate, pH 5.0, at a flow rate of 0.5 ml/min. Aliquots of 0.25 ml were taken for radiolabel content measurement. Samples without treatment were run as controls. Fractions of intact HS after chondroitinase ABC treatment and disaccharide fractions after combined-heparinases treatment were pooled, lyophilized and subjected to further analyses.

To analyse the size of low- and high-sulphated regions of perlecan HS from the two cell lines, HS samples from the previous steps were subjected to separate heparinase I or III treatments and were applied to a Superdex[®] Peptide HR 10/30 HPLC column. Fractions (0.5 ml) were collected and counted for radioactivity in order to characterize heparinase I and III resistant regions (low- and high-sulphated regions respectively).

Disaccharides derived from combined heparinase I and III treatments were analysed by strong anion-exchange chromatography (SAX)-HPLC. Lyophilized [^3H]HS disaccharides of both JB6 and RT101 perlecan were resuspended separately in 200 μl of 28 mM KH_2PO_4 , pH 4.6. The samples were analysed on a 0.4 cm $\times$ 25 cm Partisil SAX column (Whatman Inc, Clifton, NJ, U.S.A.) using step gradients with increasing concentrations of 28 mM, 150 mM and 400 mM KH_2PO_4 buffers [29,30]. The samples were run at 1 ml/min and fractions (1 ml each) were collected for radiolabel measurement. The elution peaks were identified by comparison of their retention times with those of heparin disaccharide standards (Sigma, St. Louis, MO, U.S.A.).

Column calibration

The Superdex HR10/30 column was calibrated with nitrous acid-generated [^3H]HS oligosaccharides. Elution positions of dioctasaccharides were verified with individual oligosaccharides purified by gel filtration on a Bio-Gel P-10 column. Excluded (V_0) and total volume (V_t) were determined by the elution of heparin (> 10 kDa) and sucrose respectively.

Affinity co-electrophoresis analysis of HS-growth-factor interactions

The affinities of the interactions between growth factors and HS from both JB6 and RT101 perlecan were determined by affinity

co-electrophoresis [31]. [35 S]Perlecan GAG samples dissolved in loading buffer (5 % sucrose/0.05 % Xylene Cyanol) were applied equally into horizontal slots in a 1 % (w/v) Sea Plaque[®] agarose gel (FMC Bioproducts, Rockland, ME, U.S.A.) and electrophoresed through precast 42-mm-long longitudinal lanes containing different concentrations (two-folded serial dilutions ranging from 0 to 200 nM) of different growth factors: rh (recombinant human) FGF-1, -2 (Promega, Madison, WI), rh FGF-4 (R & D Systems Inc., Minneapolis, MN), rh FGF-7 (KGF, Promega), and rh heparin-binding epidermal growth factor (HB-EGF) (R & D Systems Inc., Minneapolis, MN, U.S.A.). The gels were prepared using 50 mM Mopso [3-(*N*-morpholino)-2-hydroxypropanesulphonic acid], 125 mM sodium acetate and 0.5 % CHAPS, pH 7.0, as previously described [32,33]. The electrophoresis running buffer was identical with the buffer used to prepare gels, except CHAPS was excluded. Electrophoresis was performed at 50 V (constant voltage) for 2 h in a cooling system. Gels were dried under vacuum and subjected to autoradiography. The results of the mobility shift in this study were used to calculate the retardation coefficient (R) which is found by measuring the distance between migration of the HS in the presence of ligand (M) and the migration of the free HS (N) ($R = M/N$; [31]). The binding affinities (K_d ; slope $\approx -1/K_d$) were calculated from Scatchard plots of $R/[growth\ factor]_{free}$ against R [31,34] using PSI-Plot version 5.5 (Poly Software International, Sandy, UT, U.S.A.). The R values were determined as means from at least two repeated experiments.

RESULTS

Increased expression of perlecan in tumorigenic cells

The expression of perlecan mRNA by transformed RT101 cells compared with that of wild-type JB6 cells was first examined by using an RNase protection assay (Figure 1A). The expected fragments (≈ 174 bp) of perlecan transcript were detected in both JB6 (lanes 1) and RT101 (lanes 2) samples. Quantification of the expression level by densitometric analysis indicated up to a 5–7-fold increase in perlecan mRNA in tumorigenic cells. This was confirmed by RT-PCR analysis (Figure 1B). Increased message was detected in RT101 samples (Figure 1B, lanes 2 and

Figure 1 Increased perlecan mRNA expression in tumorigenic cells

Ribonuclease protection assay (A) of JB6 and RT101 samples and RT-PCR (B) were analysed for perlecan mRNA levels. (A) Perlecan mRNAs from total RNA preparation of JB6 (lane 1) and RT101 (lane 2) cells were separated on denaturing 5%-polyacrylamide gel after hybridization with 32 P-labelled probe and ribonuclease treatment. The arrowhead ($\blacktriangleright$) indicates the 174 bp protected product. (B) Amplification products of 550 bp were detected by RT-PCR analysis. JB6 samples, lanes 1, 3 and 5; RT101 samples, lanes 2, 4 and 6. Std, standard marker. β -Actin was used to normalize the amplified products (lanes 3–6, arrowhead). Perlecan primers were omitted in lanes 5 and 6.

Figure 2 Increased perlecan protein levels in tumorigenic cells

Slot-blot of perlecan from both JB6 and RT101 cells, probed with H5L5, a rat monoclonal antibody against perlecan domain III. (A) Aliquots of serial dilutions of total PGs normalized by cell number. (B) Aliquots of serial dilutions normalized to the total amount of secreted PG. Serum-containing culture medium was used as a control.

Figure 3 Perlecan is substituted with HS in JB6 cells, but in RT101 cells some bears CS

Immunoblotting of perlecan secreted by JB6 and RT101 cells with polyclonal antibody Ey#10 (A) against perlecan core protein, and 1B5 (B) monoclonal antibody against chondroitin stubs detected after chondroitinase ABC treatment. In each blot, the total PG pool samples were untreated (lanes 1), heparinase III-pretreated (lanes 2), heparinase III- and chondroitinase ABC-pretreated (lanes 3), or chondroitinase ABC-pretreated (lanes 4). Small arrows denote molecular-mass standards of 200, 97 and 68 kDa in (A) and 200, 120, 84 and 52 kDa in (B). A discrete perlecan core protein is visible in both cell types after heparinase III treatment (long arrow in A), with a very small amount of core protein resolved after chondroitinase ABC treatment in lane 4 of RT101 cells. The presence of multiple CSPGs in both cell types is shown in (B), after chondroitinase ABC treatment. The double-ended arrows show the presence of CS-substituted perlecan in RT101, but not JB6, cells. E, enzymes only without proteoglycan.

Figure 4 Perlecan GAG analysis

Superdex® Peptide HR 10/30 HPLC elution profiles of intact ^3H -labelled GAGs (**A**, **D**), heparinase III-pretreated GAGs (**B**, **E**) and chondroitinase ABC-pretreated GAGs (**C**, **F**). (**A**), (**B**), and (**C**), JB6 GAGs; (**D**), (**E**) and (**F**), RT101 GAGs. Fractions after V_0 are shown and the V_t is fraction 40. Some material in RT101 cells is resistant to heparinase III (**E**) and is sensitive (**F**) to chondroitinase ABC (arrows).

Figure 5 Comparison of the size of perlecan GAGs synthesized by JB6 and RT101 cells

$^{35}\text{SO}_4$ -labelled GAGs were released from perlecan core protein by alkaline borohydride treatment, and chromatographed on a TSK 4000 HPLC column. Aliquots of each fraction were analysed by liquid-scintillation counting and compared with polysaccharide standards of known mass. Fractions after V_0 are shown.

4) compared with that of the wild-type JB6 samples (Figure 1B, lanes 1 and 3) when normalized to β -actin levels (Figure 1B, lanes 3–6). A single transcript of ≈ 14.5 kb was detected in both cell

types by Northern-blot analysis (results not shown), indicating a lack of alternatively spliced variant forms in these cell types. In addition, slot-blot analysis of perlecan core protein, either normalized to cell number (Figure 2A) or total PG concentration (Figure 2B), showed a 3–4-fold increase in the RT101 samples when detected with a rat monoclonal antibody specific to perlecan domain III.

Tumour-cell-derived perlecan contains CS/DS GAGs

Perlecan is predominantly expressed as an HSPG, but in some circumstances it can be substituted with CS/DS GAGs [18–20]. To identify the type of GAGs on perlecan produced by both cell types, perlecan was purified from conditioned media of both wild-type JB6 and transformed RT101 cells. Gel electrophoresis and immunoblotting with a polyclonal core-protein-specific antibody (Figure 3A), and monoclonal antibodies against chondroitin stubs (Figure 3B) revealed a polypeptide of molecular mass ≈ 400 kDa after heparinase III digestion in samples from JB6 and RT101 cells (Figure 3A). A small amount of core protein after chondroitinase ABC treatment was seen in RT101, but not JB6, cells. The presence of galactosaminoglycan on the core protein of the RT101-derived perlecan was shown by monoclonal antibodies detecting unsaturated uronic acid-con-

Figure 6 Comparison of overall sulphation levels of perlecan GAGs

Gradient anion-exchange chromatography of $^{35}\text{SO}_4$ -labelled intact perlecan from both JB6 and RT101 cells. The samples, with or without chondroitinase ABC treatment, were applied to DEAE-Sephacel columns. The columns were washed and eluted with a linear gradient of 0.2–1.5 M salt. Fractions were collected and analysed for conductivity and radiolabel. C'ABC, chondroitinase ABC-pretreated.

taining stubs resulting from chondroitin ABC treatment (Figure 3B). No perlecan core protein was seen under equivalent conditions in JB6 cells (Figure 3B). However, a number of unrelated, lower-molecular-mass CSPG/DSPG core proteins were detected in both JB6 and RT101 preparations. The presence of galactosaminoglycan in purified RT101 perlecan was also confirmed by analysis of metabolically labelled samples. JB6- and RT101-derived GAGs were eluted in the V_0 of a Superdex HR10/30 column as a single population (Figures 4A and 4D). Heparinase III treatments (Figures 4B and 4E) degraded JB6 and most of the RT101 sample to a mixture of short oligosaccharides and a prominent disaccharide fraction. However, the presence of intact GAG in the RT101 sample (Figure 4E, arrow) indicated the presence of CS/DS. To confirm this, samples were also treated with chondroitinase ABC. Whereas the JB6 GAGs were not sensitive to chondroitinase ABC treatment, a subpopulation of RT101-derived GAGs (< 25%) were degraded (Figure 4F, arrows), confirming the presence of CS/DS on the core protein of the RT101-derived perlecan, but not that of the non-transformed JB6 counterpart.

Structural analysis of GAG chains on perlecan of both cell types

For size analysis, GAGs were released from immunopurified perlecan by alkaline elimination and resolved by gel-filtration HPLC on a TSK 4000 column (Figure 5). Both samples were eluted at a similar K_{av} (≈ 60 kDa), indicating that there was no

difference in size of the GAG chains on perlecan produced by either cell type. The relative sulphation level of perlecan GAG was determined by gradient anion-exchange chromatography (Figure 6). ^{35}S -radiolabelled intact perlecan from both JB6 and RT101 cells was eluted at the same position from the DEAE-Sephacel columns (Figures 6A and 6B). To evaluate the influence of CS/DS chains on RT101 derived perlecan, some of the samples were also pretreated with chondroitinase ABC. This treatment did not change the elution profile of the JB6 perlecan (cf. Figures 6A and Figure 6C), consistent with the absence of CS/DS. However, removal of CS/DS by chondroitinase ABC altered the net charge of the RT101 perlecan, which was then eluted at a lower salt concentration (cf. Figures 6B and 6D). The HS of the RT101 perlecan was, therefore, less sulphated than that of the JB6 cells, but the additional presence of CS/DS resulted in a similar overall sulphation. This data also confirmed that the RT101 perlecan contained CS/DS chains.

Total disaccharide compositions of perlecan HS

Changes in the sulphation level suggested that HS from wild-type and tumour-cell perlecan might have different disaccharide compositions. To test this, [^3H]glucosamine-labelled HS from both JB6 and RT101 perlecan were depolymerized with combined heparinase I and III treatment. The disaccharides purified by gel-filtration HPLC were resolved by SAX HPLC (Figure 7 and Table 1). Major peaks identified by comparison with heparin-

Figure 7 SAX HPLC analyses of ^3H -labelled HS disaccharides derived from JB6 (A) and RT101 (B) cells

(A) and (B) show the separation of disaccharides resulted by depolymerization of HS using combined heparinases I and III. The elution of heparin disaccharide standards was as follows: peak 1, $\Delta\text{UA-GlcNAc}$; peak 2, $\Delta\text{UA-GlcNS}$; peak 3, $\Delta\text{UA-GlcNAc(6S)}$; peak 4, $\Delta\text{UA(2S)-GlcNAc}$; peak 5, $\Delta\text{UA-GlcNS(6S)}$; peak 6, $\Delta\text{UA(2S)-GlcNS}$; peak 7, $\Delta\text{UA(2S)-GlcNAc(6S)}$; and peak 8, $\Delta\text{UA(2S)-GlcNS(6S)}$.

Table 1 Comparative disaccharide compositions of HS from JB6 and RT101 perlecan

HS samples were degraded by combined heparinase I and III digestion. The resulting disaccharides were analysed by SAX-HPLC. The results represent the mean values obtained from two determinations.

Disaccharide subunits	Cells ...	Composition (%)	
		JB6	RT101
(1) $\Delta\text{UA-GlcNAc}$		52	58
(2) $\Delta\text{UA-GlcNS}$		22	21
(3) $\Delta\text{UA-GlcNAc(6S)}$		4	3
(4) $\Delta\text{UA(2S)-GlcNAc}$		2	1
(5) $\Delta\text{UA-GlcNS(6S)}$		2.5	3
(6) $\Delta\text{UA(2S)-GlcNS}$		10.5	9.5
(7) $\Delta\text{UA(2)-GlcNAc(6S)}$		1	0.5
(8) $\Delta\text{UA(2S)-GlcNS(6S)}$		6.5	3
N-S (%) ...		41	37
2-O-S (%) ...		20	15
6-O-S (%) ...		14	10
No. of sulphate groups/100 disaccharides ...		75	62

derived disaccharide standards were quantified (Table 1). The major disaccharide of both HS species was unsaturated uronic acid-*N*-acetylglucosamine ($\Delta\text{UA-GlcNAc}$), which comprised over half of the total disaccharide units. Unsaturated uronic acid-*N*-sulphated glucosamine ($\Delta\text{UA-GlcNSO}_3$) was a second prominent constituent in both cases. The level of *N*-sulphation was similar for wild-type and tumorigenic cells (41 and 37% respectively). Consistent with the anion-exchange-chromatography results, progression to the tumorigenic phenotype was associated with a decrease in sulphation; the average number of

sulphate groups/100 disaccharides in the JB6 and RT101 perlecan HS were 75 and 62 respectively. In particular, perlecan HS from RT101 cells had lower levels of 2-O- and 6-O-sulphation, and increased amount of $\Delta\text{UA-GlcNAc}$ compared with the wild-type. On the basis of the decrease of the trisulphated disaccharide unsaturated uronic acid (2-sulphate)-*N*-sulphated glucosamine 6-sulphate [$\Delta\text{UA(2S)-GlcNS(6S)}$] in perlecan from tumorigenic cells, the change in sulphation level mostly affected the highly sulphated S-domains, where this disaccharide can be primarily found.

Analysis of heparinase I and III-resistant regions on perlecan HS

In general, HS chains comprise of spatially discrete highly sulphated, iduronate-rich domains (S-domain) interspersed with larger regions of unmodified domains (U-domain). The boundary between S- and U-domains contains short mixed (M) sequences where *N*-acetylated disaccharides alternate with *N*-sulphated ones [35]. Both the composition and spacing of the domains is important for HS-ligand-binding abilities and can be different for HS derived from different sources. To identify these regions on HS of JB6- and RT101-derived perlecan, HS samples were digested either with heparinase I {cleaves predominantly the sequence iduronic acid (2-sulphate)-*N*-sulphated glucosamine [$\text{IdoUA(2S)-GlcNS} \pm (6\text{S})$] in highly sulphated regions} or heparinase III {cleaves predominantly the sequence glucuronic acid-*N*-acetylglucosamine (± 6 -sulphate) [$\text{GlcUA-GlcNA} \pm (6\text{S})$] in unmodified regions} and the products were resolved on Superdex® Peptide 10/30 HPLC column. Heparinase I treatment generated two classes of products, which were similar in size and composition for the JB6 and RT101 perlecan HS (Table 2). Over 80% of the products were longer resistant fragments originating from intact U-domains and flanking regions resistant to the enzyme. These were 14–20 sugar residues in length, and the rest was a mixture of shorter oligosaccharides from the susceptible regions containing 2-O-sulphated hexuronic acid, which primarily occur in S-domains (Table 2). There was no clear decrease in the short oligosaccharides from tumorigenic RT101-cell perlecan HS, although the decrease in 2-O-sulphation in perlecan HS from these cells could be expected to alter the susceptibility to heparinase I. However, it is possible that moderate amounts of radiolabelled immunoprecipitated perlecan HS, which were available for the analysis, were not sufficient to permit the sensitive detection of small changes in the short-product amount.

Heparinase III degrades predominantly U-domains, and therefore can be used to determine the size of S-domains, which remain intact after the treatment. These resistant S-domains were on average octasaccharides for JB6 and hexaoctasaccharides for RT101 (Table 2). SAX analysis detected lower sulphation levels and more *N*-acetylated glucosamine in perlecan HS from RT101 cells, which should lead to higher susceptibility to heparinase III. Consistently, the amount of heparinase III-generated disaccharides is higher for the RT101 sample (Table 2), indicating that the tumorigenic RT101-cell-derived perlecan contains HS with longer or more U-domains. These profiles were consistent with the SAX-HPLC profiles (Figure 7 and Table 1). Combining these results, it appears that the HS of RT101 consists of slightly smaller S-domains, but slightly larger and/or an increased number of U-domains compared with those of the JB6 wild-type.

Binding of perlecan-derived HS to growth factors

The data suggest that structural differences in HS are associated with the process of tumorigenic transformation in these murine

Table 2 Susceptibility of perlecan HS to heparinases I and III

Perlecan HS was degraded by either heparinase I or III and subjected to Superdex® Peptide HR 10/30 HPLC. The results represent the mean values from three sets of data for heparinase I treatment and two sets of data for heparinase III treatment. The heparinase III-treatment values are means only, since S.D. is not valid for $n = 2$. Abbreviation: fr, fraction.

Enzyme	Cells ...	Percentage of total pool of products generated by heparinase I or III treatments (mean $\pm$ S.D.)	
		JB6	RT101
Heparinase I			
14–20 Sugars (fr 1–16)		80.3 $\pm$ 6.0	84.9 $\pm$ 0.8
4–12 Sugars (fr 17–30)		2.2 $\pm$ 3.4	9.0 $\pm$ 1.8
Disaccharides (fr 31–40)		7.5 $\pm$ 2.7	6.1 $\pm$ 1.1
Average size of the HI-resistant domain (U-domain) ...		$\approx$ 20 Sugar residues (is eluted at fraction 7)	$\approx$ 20 Sugar residues
Heparinase III			
4–14 Sugars		42.1	35.4
Disaccharides		57.8	64.5
Average size of the HIII-resistant domain (S-domain) ...		$\approx$ 8 Sugar residues (is eluted at fraction 21)	6–8 Sugar residues (is eluted at fraction 22)

epidermal cells. Potentially, these changes could affect the HS ability to bind various ligands. Therefore, we used affinity co-electrophoresis to assess the interaction between perlecan-derived HS from both JB6 and RT101 cells to several growth factors involved in regulation of epithelial-cell growth. Four members of the FGF family, namely FGF-1, -2, -4 and -7, and HB-EGF, were examined. Affinity co-electrophoresis measures relative retardation of radiolabelled HS in the presence of various ligand concentrations, which represents the ligand-bound fraction of HS, and therefore can be used to calculate affinity. Dissociation constants for the complex of HS and various growth factors were determined by Scatchard analysis. The Scatchard plots and corresponding dissociation constants are shown in Figure 8. The R values were averaged from the results of at least two independent experiments. Over the range of tested concentrations (12–200 nM), perlecan HS from both cell types bound with high affinity to all growth factors. However, HS from RT101 perlecan bound with slightly higher affinity to FGF-1 ($K_d \approx 40$ versus 66.7 nM), FGF-2 ($K_d \approx 12.5$ versus 29.2 nM), FGF-7 ($K_d \approx 29.8$ versus 48.8 nM) and HB-EGF ($K_d \approx 26.4$ versus 35.8 nM), but with a marginally lower affinity to FGF-4 ($K_d \approx 40.2$ versus 28.8 nM) compared with the JB6-derived perlecan HS.

DISCUSSION

Perlecan is a widespread basement-membrane and extracellular-matrix PG. It is among the most complex of extracellular-matrix molecules, having a multidomain core protein of approx. 400 kDa, substituted with GAG chains, which may be HS and sometimes CS/DS [12]. The substitution of the core protein is most commonly seen on the N-terminal domain I, but has also been recorded on the C-terminal domain V [27,36]. Virtually all basement membranes possess perlecan, though interestingly, where the gene has been deleted, as recently reported [37], the major impact appears to be on skeletal tissues. This is perhaps because perlecan is a component of cartilage [38]. In addition, perlecan has been suggested to be an important component in tumour biology; it may form a scaffold for cellular interactions, since the core protein can interact with integrins [12]. An additional important feature of perlecan is that its HS chains may bind many growth factors, including some that are potent inducers of angiogenesis [15]. It has been suggested that tumour-released matrix metalloproteinases (MMPs) such as stromelysin (MMP-3) and collagenase 3 (MMP-13) may cleave perlecan core protein to release GAG chains with attached growth factors [16]

that promote angiogenic responses. Therefore, it is important to understand the biology of perlecan in the context of tumour progression.

The model we have used allows a direct comparison of a wild-type epidermal-cell line with its transformed counterpart. The JB6 epidermal cell line is non-tumorigenic, but when exposed to phorbol esters, is irreversibly transformed [25,26]. We have compared the structure of perlecan from these two cell lines as a first stage in ascertaining whether this molecule may be important in epidermal tumorigenesis. The major differences seen between JB6 and RT101 perlecan were as follows. First, both the mRNA and protein levels of perlecan core protein were increased in the transformed cell line, in agreement with previous reports showing that other transformed cells can be prolific producers of this matrix PG [10,11]. Secondly, while GAG chain size does not differ between the two cell types, the presence of a small amount of galactosaminoglycan was detected in the transformed, but not the wild-type, epidermal cells. The presence of CS and/or DS on perlecan has been noted several times, and this is one of several PGs that can be synthesized as a hybrid PG [12,19]. The impact of alternate glycanation on the biology of perlecan is not understood. For instance, it is not known whether the interaction of growth factors or other HS-binding moieties is influenced by the presence of galactosaminoglycan. In some cases, DS does possess growth-factor-binding capabilities similar to, but usually with lower affinity than, those seen with the equivalent interaction with HS [39,40]. In the present study, however, it appeared that the presence of hybrid perlecan molecules correlated with the transformed state. This may represent a case where the increased synthesis of perlecan leads to some of the glycanation being of the 'default' pathway, i.e., galactosaminoglycan. Similar cases have been reported previously, but not directly in the context of transformation [41].

In keeping with the increased synthetic rate of perlecan, we also determined that the HS chains of RT101 perlecan were of lower overall sulphation than their normal counterparts. However, this difference is subtle, and is even masked where the CS/DS are present. Under these circumstances the perlecan from wild-type and transformed mouse epidermal cells was eluted equivalently in gradient anion-exchange chromatography. When the galactosaminoglycans were removed by chondroitinase ABC treatment, the lower sulphation of the HS chains became apparent. Consistent with these data, we ascertained that there were small differences in the fine structure of the HS synthesized by JB6, compared with RT101, cells. The RT101-derived perlecan

Figure 8 Scatchard analyses of perlecan HS binding to growth factors

K_d values were calculated from the slopes (slope = $-1/K_d$) of the fitted straight lines using the PSI-Plot program. The y-axis represents the values of $R/[growth\ factor]$ (in nM) and the x-axis is the relative retardation, R . The R values are the averages from at least two repeated experiments (see the Experimental section). (A) and (F), FGF-1; (B) and (G), FGF-2; (C) and (H), FGF-4; (D) and (I), FGF-7; and (E) and (J), HB-EGF. JB6-derived perlecan HS is shown in (A)–(E), and RT101-derived perlecan HS is shown in (F)–(J).

HS had decreased 2-O- and 6-O-sulphation with a concomitant increase in unsulphated disaccharides.

Our results have some similarity to a recent comparison of colon adenoma and carcinoma HS fine structure [5]. The latter

had HS with one-third less 2-O-sulphate and one-fifth less N-sulphate. In our study, N-sulphation was virtually unchanged, but there was around 25% less 2-O-sulphation. Our studies also revealed a similar decrease in 6-O-sulphation, a modification that

was seen to increase in the previous study [5]. The reasons are unclear and may relate to tissue-specific expression of sulphotransferases. In addition, our study is distinct from previous work, concentrating on one PG, perlecan, rather than total HS. Despite some reduction in sulphation, further analysis showed that the affinity of the HS chains for several growth factors, was not diminished. Four members of the FGF family were chosen for this study, all of which have been implicated in epithelial tumorigenesis [42,43]. In the case of the FGF-7, these interactions may not be restricted to the HS chains, but may also extend to an involvement of the perlecan core protein [13]. However, in no case was a substantial difference in affinity seen in the interactions of these four members of the FGF family or HB-EGF with perlecan from normal and transformed cells. The affinities are high, in the nanomolar range, and therefore indicate that perlecan of both cell types has the ability to bind these potent growth, differentiation and angiogenesis-promoting factors. This is the first time that perlecan-derived HS has been shown to interact with FGF-4 and HB-EGF. Future *in vivo* work will determine just how important the presence of perlecan in the tumour matrix is, especially given its potential abundance and binding abilities. Recent exciting data show that antisense targeting of perlecan can block both tumour growth and angiogenesis *in vivo* [13], so it may well be that detailed understanding of this complex PG will be highly relevant for future understanding of tumour invasiveness.

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