A rapid quantitative assay for the detection of mammalian heparanase activity

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Heparan sulphate (HS) is an important component of the extracellular matrix and the vasculature basal laminar which functions as a barrier to the extravasation of metastatic and inflammatory cells. Cleavage of HS by endoglycosidase or heparanase activity produced by invading cells may assist in the disassembly of the extracellular matrix and basal laminar, and thereby facilitate cell migration. Heparanase activity has previously been shown to be related to the metastatic potential of murine and human melanoma cell lines [Nakajima, Irimura and Nicolson (1988) *J. Cell. Biochem.* **36**, 157–167]. To determine heparanase activity, porcine mucosal HS was partially de-N-acetylated and re-N-acetylated with [³H]acetic anhydride to yield a radiolabelled substrate. This procedure prevented the masking of, or possible formation of, new heparanase-sensitive cleavage sites as has been observed with previous methods of radiolabelling. Heparanase activity in a variety of tissues and cell homogenates including human platelets, colonic carcinoma cells, umbilical vein endothelial cells and rat mammary adenocarcinoma cells (both metastatic and non-metastatic variants) and liver homogenates all degraded the substrate in a stepwise fashion from 18.5 to approximately 13, 8 and finally to 4.5 kDa frag-

ments, as assessed by gel-filtration analysis, confirming the substrate as suitable for the detection of heparanase activity present in a variety of cells and tissues. A rapid quantitative assay was developed with the HS substrate using a novel method for separating degradation products from the substrate by taking advantage of the decreased affinity of the heparanase-cleaved products for the HS-binding plasma protein chicken histidine-rich glycoprotein (cHRG). Incubation mixtures were applied to cHRG–Sepharose columns, with unbound material corresponding to heparanase-degradation products. Heparanase activity was determined for a variety of human, rat and murine cell and tissue homogenates. The highly metastatic rat mammary adenocarcinoma and murine lung carcinoma cell lines had four to ten times the heparanase activity of non-metastatic variants, confirming the correlation of heparanase activity with metastatic potential. Human cancer patients had twice the serum heparanase levels of normal healthy adults. The assay will be valuable for the determination of heparanase activity from a variety of tissue and cell sources, as a diagnostic tool for the determination of heparanase potential, and for the development of specific inhibitors of heparanase activity and metastasis.

INTRODUCTION

An important process in tissue invasion by blood-borne malignant tumour cells and leukocytes involves their passage through the vascular endothelial cell layer and subsequent degradation of the underlying basal lamina and extracellular matrix (ECM) with a battery of secreted and/or cell surface protease and glycosidase activities [1–3]. The basal lamina and underlying connective-tissue stroma consist predominantly of a complex network of fibronectin, laminin, collagen type IV and vitronectin, each of which interact with heparan sulphate (HS) side chains of heparan sulphate proteoglycans (HSPG) embedded within the matrix [4]. HS chains generally consist of clusters of sulphated disaccharide units (predominately N-sulphated glucosamine linked 1 → 4 to α-L-iduronic acid residues) separated by lowly or non-sulphated regions (predominantly disaccharide units of N-acetylated glucosamine linked 1 → 4 to β-D-glucuronic acid) [5,6]. Cleavage of the HS chains by endoglycosidase or heparanase activity produced by invading cells may therefore assist in the disassembly of the ECM and facilitate cell migration. Heparanase activity has been shown to be related to the

metastatic potential of murine and human fibrosarcoma and melanoma cell lines [1,7–10]. Furthermore heparanase activity has been described in a number of tissues and cell types including rat liver [11,12], human placenta [13,14], human platelets [15–17], cultured human skin fibroblasts [18], human neutrophils [19,20], activated but not resting rat T-lymphocytes [21], normal and neoplastic murine B-lymphocytes [22], human monocytes [23] and human umbilical vein endothelial cells (HUVECs) [24,25]. Because the cleavage of HS appears to be essential for the passage of metastatic tumour cells and leukocytes through basement membranes, studies of heparanase inhibitors provides the potential of developing a novel and highly selective class of anti-metastatic and anti-inflammatory drugs [3,26–29].

To date, three separate heparanase activities have been reported: mouse melanoma B16 heparanase, which cleaves HS only; human platelet heparanase, which cleaves both heparin and HS; and a mouse mastocytoma endoglucuronidase, which was reported to cleave newly synthesized heparin precursor but not heparin or HS [9,15,30]. In order to purify and characterize mammalian heparanase activities and to screen for and develop effective heparanase inhibitors, a simple and rapid assay for

Abbreviations used: bFGF, basic fibroblast growth factor; cHRG, chicken histidine-rich glycoprotein; ECM, extracellular matrix; GlcA, glucuronic acid; GlcNAc, N-acetylated glucosamine; GlcNS, N-sulphated glucosamine; GlcNS3S, glucosamine N,3-disulphate; hHRG, human histidine-rich glycoprotein; HRG, histidine-rich glycoprotein; HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; HUVECs, human umbilical-vein endothelial cells; IdoA2S, iduronic acid 2-sulphate.

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heparanase activity is required. However, previous heparanase assays have been cumbersome and time-consuming in both preparation of the radiolabelled substrate and separation of degradative products from the uncleaved substrate. Frequently heparanase assays have involved the biosynthetic radiolabelling of ECM-associated HSPG and the detection of HS chain degradation by gel-filtration analysis of radiolabelled material released from the ECM [3,24]. Such an approach suffers from the main disadvantage that degradation of HS chains in an ECM involves the synergistic action of proteases which are required to expose the HS chains for subsequent heparanase attack [31–34]. Furthermore most heparanase assays have required extensive degradation of the radiolabelled HS (or ECM-derived HSPG) substrate to allow separation of the degraded product from the substrate by gel filtration [3,7,13,24], although cleavage of HS chains at a single site may be all that is required to allow passage of leukocytes and tumour cells through the basement membrane. Solid-phase heparanase assays have also been developed where chemically and biosynthetically radiolabelled heparin and HS chains were attached to a solid support, with release of radiolabel from the solid support being a measure of enzyme activity [7,17,23]. Such assays, however, suffer from the disadvantage that the immobilized substrate may be less accessible to the mammalian heparanase enzyme, and the coupling of the radiolabelled substrate to the solid support, via the substrate's reducing terminus, is a complex and inefficient procedure. Previous studies have also radiolabelled both heparin and HS by iodination at naturally occurring glucosamine residues [17] or by N-acetylation of the partially de-N-sulphated substrate [7]. Such procedures, however, may result in the masking or creation of new heparanase-cleavage sites.

We now describe a new, simple and rapid quantitative assay for the detection of mammalian heparanase activity towards a natural radiolabelled substrate, which can detect the minimal number of HS chain cleavages that are likely to occur *in vivo*. The assay is based on a novel principle, namely the loss of a protein-binding site from HS chains after chain cleavage. In the assay, advantage is taken of an initial observation that the HS-binding plasma protein histidine-rich glycoprotein (HRG) masks heparanase-cleavage sites on HS chains. After heparanase digestion, radiolabelled HS fragments (products) are not bound by mini-columns of HRG-coupled Sepharose beads, unlike the remaining intact and partially degraded substrate, allowing a rapid separation of the cleaved product from the substrate.

MATERIALS AND METHODS

Materials

Heparin-derived molecular-mass standards of 16.7, 10.6, 6.7 and 3.1 kDa were a gift from Nova Nordisk (Gentofte, Denmark) [35]. Porcine mucosal HS (ORG 553) was a gift from Organon Int. Bv (Oss, The Netherlands). Pharmacia Fine Chemicals (Uppsala, Sweden) supplied PD-10 columns, DEAE-Sepharose and CNBr-activated Sepharose 4B. Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied crystalline BSA, bovine lung heparin, chondroitin ABC lyase, hydrazine hydrate and hydrazine sulphate. [³H]Acetic anhydride (500 mCi/mmol) in toluene solution was obtained from Amersham International (Sydney, Australia). Beckmann (Gladsville, Australia) supplied Ready Safe scintillation fluid. Dowex AG50W (X8, H⁺ form; 100–200 mesh) was obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.).

The following cell lines were cultured and harvested as previously described: the metastatic human colonic carcinomas HCT 116 [36] and KM 12 SM [37]; the metastatic murine

melanoma B16-F10, the highly metastatic 13762 MAT and DMBA-8A rat mammary adenocarcinoma, and their non-metastatic variants J-clone and DMBA-Sask [27]; HUVECs and the macrophage cell line U937 [24]. The human keratinocyte cell line (KJD) was an ATCC (Rockville, MA, U.S.A.) cell line, CRL 1485 [38]. Human platelets were obtained by the method of Bartlett et al. [24]. Cultured human lung fibroblasts were a gift from Ms June Hornby, J.C.S.M.R., Canberra, Australia. Human mammary arteria smooth-muscle cells were a gift from Dr. Peter Little, The Baker Institute, Melbourne, Australia [39]. The murine Lewis lung carcinoma metastatic (D122) and non-metastatic (LLC) variant cell lines were a gift from Dr. James W. Dennis, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada, and the murine lung carcinoma metastatic (A3a) and non-metastatic (SP1) variant was a gift from Dr. Lea Eisenbach, Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel. Cell pellets from these cell lines were supplied frozen (–80 °C) by Dr. Robin Anderson, Peter MacCallum Cancer Institute, Melbourne, Australia. Cell homogenates were prepared by suspending 10⁶ cells or 10⁸ platelets (which had been previously washed in normal saline) in 0.2 ml of aq. 0.1 % (v/v) Triton X-100 and disrupting by freezing and thawing three times in a solid CO₂/ethanol mixture.

Freshly excised livers from 10-week-old Fischer F344 rats were homogenized in 3 vol. (w/v) of 15 mM dimethyl glutarate buffer, pH 6.0, containing 0.5 M NaCl using a Polytron homogenizer. Serum and both tumorigenic and adjacent normal lung tissue from human cancer patients was obtained frozen (–80 °C) from the Royal North Shore Hospital, Sydney, Australia. The tissue was thawed and homogenized as described above. Frozen serum from cancer patients and healthy volunteers was thawed and dialysed for 16 h at 4 °C against normal saline. Protein concentrations were estimated by a Bio-Rad Coomassie protein assay kit (Bio-Rad Laboratories) using BSA as a standard.

Preparation of radiolabelled HS

Crude porcine intestinal mucosal HS ORG 553 (100 mg) was dissolved in 2 ml of water, dialysed for 4 h against 2 litres of normal saline and for 16 h against 2 litres of water at 4 °C in dialysis tubing with a 10 kDa cut-off. The dialysed solution was mixed with 2 ml of 0.1 M Tris/HCl, pH 8.0, containing 0.1 mg/ml BSA and 2 units of chondroitin ABC lyase, and incubated at 37 °C for 16 h. After dialysis at 4 °C for 16 h against 2 litres of PBS, the solution was applied to a DEAE-Sepharose column (1.0 cm × 6.0 cm) equilibrated in PBS. The column was washed successively with PBS (12 ml) and 0.3 M NaCl (12 ml) before being eluted with 10 ml of 2 M NaCl which was subsequently lyophilized. The solid was dissolved in the minimum amount of water, and desalted using a Pharmacia PD-10 column. The eluate appearing ahead of the NaCl (detected by addition of a drop of a 20 % silver nitrate solution) was lyophilized to yield 33 mg of purified HS. The purified HS (26 mg) was dissolved in hydrazine hydrate (1 ml) containing hydrazine sulphate (20 mg), and the mixture heated at 100 °C for 2 h in a capped glass vial. The mixture was cooled and dried under a stream of nitrogen. Toluene (1 ml) was added and the mixture dried; this procedure was repeated twice more. The residue was dissolved in 2 ml of 1 M NaCl, desalted on a PD-10 column, and the desalted material passed through a Dowex 50 (X-8; Na⁺ form) column (1.0 cm × 6.0 cm) which was washed with a further 10 ml of water. The combined flow through and washings were lyophilized to yield 19 mg of partially de-N-acetylated HS. The de-N-acetylated HS (19 mg) was dissolved in 1 ml of 0.5 M NaHCO₃,

containing 10% (v/v) methanol, cooled to 0 °C in an ice bath and 0.1 ml of [³H]acetic anhydride (5 mCi; 500 mCi/mmol) in toluene added, and the mixture stirred vigorously for 3 h at 0 °C. A further 0.05 ml of methanol, 0.1 ml of Na₂CO₃ and 0.05 ml of acetic anhydride were added with stirring for 30 min at 0 °C. This procedure was repeated twice more, checking that the pH remained alkaline before the mixture was acidified to pH 4.0 with acetic acid. After the mixture was warmed to 22 °C, the toluene was removed under a stream of nitrogen and the solution desalted on a PD-10 column equilibrated and developed in aq. 10% (v/v) ethanol. Radioactive material appearing ahead of the [³H]acetate peak was lyophilized to yield 21 mg of radiolabelled HS which was dissolved in aq. 10% (v/v) ethanol at a concentration of 2 mg/ml and stored at -20 °C.

Preparation of HRG-Sepharose

Chicken histidine-rich glycoprotein (cHRG) was prepared from chicken plasma by phosphocellulose ion-exchange chromatography [40]. The purity of the preparation was demonstrated by SDS/PAGE where a single Coomassie Blue-stained band with a molecular mass of 135 kDa was detected. The material was concentrated to a protein concentration of 2 mg/ml, dialysed against PBS and stored at -20 °C until use. Bovine lung heparin (50 mg) was dissolved in 10 ml of 0.5 M NaHCO₃ containing 10% (v/v) methanol and cooled to 4 °C before five aliquots of 0.04 ml of acetic anhydride were added at 5 min intervals. The pH was kept above 8.0 with 1 M Na₂CO₃. The mixture was stirred for a further 30 min before the acetylated heparin was dialysed for 16 h at 4 °C against 5 litres 0.1 M NaHCO₃ containing 0.15 M NaCl (coupling buffer).

cHRG (10 mg) was dialysed for 16 h at 4 °C against 2 litres of coupling buffer and mixed at 4 °C for 30 min with the acetylated heparin solution prepared above to prevent coupling of the HS-binding site of cHRG to the Sepharose beads. CNBr-activated Sepharose 4B (2 g) was swollen in 50 ml of 1 mM HCl for 30 min and washed with 150 ml of 1 mM HCl in a sintered funnel under vacuum before being washed with 20 ml of coupling buffer. The washed CNBr-activated Sepharose was immediately added to the heparin/cHRG solution and gently mixed for 24 h at 4 °C. A 4 ml solution of 2 M ethanolamine in coupling buffer (adjusted to pH 8.0 with 1 M HCl) was added and gently mixed for a further 16 h. The slurry was poured into a chromatography column (1.5 × 5.0 cm) and washed successively at 4 °C with 50 ml each of coupling buffer and 0.05 M Tris/HCl buffer, pH 8.0, containing 2 M NaCl and finally PBS. cHRG-Sepharose was stored in PBS at 4 °C. Coupling yielded a concentration of 0.8 mg of protein bound per ml of beads, which bound 6.2 and 3.1 nmol of radiolabelled lung heparin and HS respectively.

Heparanase assay

cHRG-Sepharose beads (0.2 ml bed column) were placed in disposable 1 ml syringes tamped with glass wool and the mini-columns placed in 10 ml centrifuge tubes standing in an ice bucket. The columns were prewashed with 1 ml of PBS before use. All column procedures were performed on ice. To assay for heparanase activity, up to 2 µl of sample was added to an incubation mixture consisting of 90 pmol of radiolabelled HS in 0.05 M sodium acetate buffer, pH 5.1, containing 5 mM *N*-acetylmannosamine and 0.1 mg/ml BSA in a total volume of 20 µl and incubated at 37 °C for the appropriate time. The reaction was stopped by freezing the tubes, 0.3 ml of cold PBS added, the solution centrifuged if necessary at 4 °C and 0.1 ml of the sample applied to a cHRG-Sepharose mini-column pre-equilibrated in PBS. The columns were washed with 0.7 ml of

PBS, the washings and flow-through material transferred to a scintillation vial, 10 ml of scintillation fluid was added, and the radioactivity determined. Enzyme activity was expressed as pmol of product formed/h per mg of protein or 10⁶ cells or ml of serum. The cHRG-Sepharose columns were regenerated by the addition of 1 ml of 0.05 M Tris/HCl buffer, pH 7.5, containing 2 M NaCl and then equilibrated with PBS.

Heparanase activity was also detected by gel-filtration chromatography of the incubation mixture on a Superose 6HR 10/30 column (Pharmacia LKB Biotechnology, Uppsala, Sweden), equilibrated in and subsequently developed in PBS. The Pharmacia FPLC system was run at a flow rate of 0.25 ml/min with 0.5 ml fractions collected directly into scintillation vials, to which 3 ml of scintillation fluid was added, and the radioactivity determined. The column was calibrated with *N*-[³H]acetylglucosamine (221 Da), bovine lung heparin (12.5 kDa) and heparin-derived molecular mass standards (16.7, 10.6, 6.7 and 3.1 kDa) which had been partially de-*N*-acetylated and re-*N*-acetylated with [³H]acetic anhydride as described above for the preparation of radiolabelled HS. Radiolabelled chondroitin 6-sulphate and carboxyl-reduced HS (prepared by the method of Karamanos et al. [41]) were also prepared by the above method.

Binding of HS to cHRG-Sepharose

Samples in 200 µl of PBS were applied to a mini-column (0.5 cm × 4.0 cm) of cHRG-Sepharose equilibrated in PBS at 4 °C. The column was washed with 2 ml of PBS and eluted with a linear gradient (2 × 10 ml) of PBS and 10 mM sodium phosphate buffer, pH 7.2, containing 1.2 M NaCl developed at 0.5 ml/min with 0.5 ml fractions collected directly into scintillation vials, to which 4 ml of scintillation fluid was added and the radioactivity determined. The columns were re-equilibrated in PBS for further use.

RESULTS AND DISCUSSION

Preparation of a radiolabelled heparanase substrate

Previous studies of heparanase activity towards heparin or HS have utilized linking reagents to permit radioiodination [17] which could mask potential cleavage sites, or acetylation of previously de-*N*-sulphated HS or heparin with radiolabelled acetic anhydride [7]. However, as was reported, the latter method both affected the apparent molecular mass of the substrate and generated heparanase-susceptible linkages in heparin, which had previously been shown to be resistant to murine B16 melanoma heparanase activity [7]. To ensure the production of a radiolabelled biologically active natural substrate for the determination of heparanase activity in a variety of biological samples, purified porcine mucosal HS was partially de-*N*-acetylated and re-*N*-acetylated with [³H]acetic anhydride to a specific radioactivity of 1650 to 2000 c.p.m./pmol, which is up to 100 times that previously reported for ¹⁴C-labelled HS [7]. This labelling procedure has the major advantage that there was unlikely to be any introduction of new or altered cleavage sites into the substrate. The substrate was stored at -20 °C in aq. 10% (v/v) ethanol where the substrate was stable for over a year without the production of appreciable degradation products despite repeated freezing and thawing. Porcine mucosal HS which has previously been isolated by Hahnenberger et al. [42] has been shown to have a peak molecular mass of 22 kDa and to contain heparitinase-resistant saccharides of 2 to 16 degrees of polymerization, enriched in IdoA2S-GlcNS sequences which mediate basic fibroblast growth factor (bFGF) activity [43]. It also contains extended HNO₂-resistant (GlcNAc-containing) se-

Figure 1 Degradation of HS as a function of incubation pH

HS was incubated with rat 13762 MAT cell homogenate at pH 4.2 (○), 5.1 (□), 6.5 (△) and 7.5 (▲) and in the absence of added enzyme at pH 5.1 (■) for 16 h at 37 °C. The incubation mixtures were fractionated by Superose 6 gel-filtration chromatography. Molecular-mass standards shown are (a) 16.7, (b) 10.6, (c) 6.7, (d) 3.1 and (e) 0.2 kDa. For full details, see the Materials and methods section.

quences of 6 to 14–16 degrees of polymerization (J. E. Turnbull, personal communication) and therefore contains alternating sulphated and non-sulphated regions typical of HS species.

Analysis of heparanase digestion of the HS substrate

Before a new heparanase assay could be developed, it was essential that the radiolabelled HS be validated as an appropriate substrate using conventional gel filtration to separate and characterize the degradation products. It was also important to define the degradative properties of the heparanase enzymes used in this study. Heparanase degradation of the radiolabelled substrate was assessed using the highly invasive rat mammary adenocarcinoma 13762 MAT and human platelet homogenates, since the tumour- and platelet-derived enzymes have previously been shown to have different substrate specificities, degrading either HS only or both heparin and HS respectively [9]. To prevent subsequent exoenzyme degradation of the heparanase-degradative products, 5 mM *N*-acetylmannosamine was added to the incubation mixture to inhibit release of ³H-labelled *N*-acetylglucosamine residues after α -*N*-acetylglucosaminidase activity [44].

HS was incubated with 13762 MAT cell homogenate in the presence of *N*-acetylmannosamine at pH 4.2, 5.1, 6.5 and 7.5 for 16 h at 37 °C, and the reaction mixture was analysed by gel-permeation chromatography (Figure 1). The sizes of the heparanase-derived products were compared with a series of radiolabelled heparin-derived molecular-mass standards and the number-average molecular mass of the products determined. HS was maximally degraded at pH 5.1 from a number-average molecular mass of 18.5 to fragments of 4.6 kDa, compared with values of 8.1 and 9.6 kDa at pH 6.5 and 7.5 respectively. The rate of degradation at pH 4.2 was slower than at pH 6.5, with a less obvious stepwise breakdown to products. Prolonged incubation of HS with 13762 MAT cell homogenate for 40 h again resulted in incomplete degradation at pH 6.5 and 7.5 (results not shown). To determine whether different enzyme activities were present or whether different cleavage sites were generated at the higher pH values, the individual products of degradation at pH 5.1, 6.5 and 7.5, after a 16 h incubation, were collected, desalted and incubated at either pH 5.1 or 6.5 with fresh homogenate for a further

Figure 2 Degradation of HS as a function of incubation time

HS was incubated with rat 13762 MAT cell homogenate at pH 5.1 for 2 (△), 4 (▲) and 16 (□) h and in the absence of added enzyme for 4 h (■) at 37 °C. The incubation mixtures were fractionated by Superose 6 gel-filtration chromatography. Molecular-mass standards a–e are as described in Figure 1. For full details, see the Materials and methods section.

16 h. In each case, each of the tested fragments was degraded to a minimum size of 4.6 kDa (results not shown). Application of the 4.6 kDa products of heparanase digestion to DEAE-Sephacrose resulted in all of the radioactivity remaining bound, even in the presence of 0.3 M NaCl, indicating that all the HS fragments resulting from heparanase digestion contained highly sulphated HS domains.

A time course incubation of 13762 MAT cell homogenates with HS at pH 5.1 for periods of 2, 4 and 16 h again demonstrated an apparent stepwise degradation of HS (Figure 2), with three apparent cleavages from 18.5 to 13.1 to 8.2 and finally to 4.3 kDa. No activity was detected towards chondroitin 6-sulphate or carboxyl-reduced HS after incubation for 16 h at pH 5.1, 6.5 or 7.5 (results not shown). Human platelet homogenate heparanase activity similarly did not act on carboxyl-reduced HS but digested HS to fragments of 4.5 kDa, which were retained by DEAE-Sephacrose in 0.3 M NaCl. Homogenates from a variety of cell lines, including the highly metastatic human colonic carcinoma HCT 116 and KM 12-SM lines, HUVECs, mouse melanoma B16-F10 cells and a rat liver extract were all demonstrated by gel-filtration analysis to hydrolyse HS to fragments with number-average molecular masses of approximately 13, 8 and finally 4.5 kDa during the course of a 16 h incubation (results not shown), indicating that the heparanase activities in each cell line were cleaving HS to similarly sized products. Previously human platelet, HUVEC, lymphocyte, neutrophil and leukocyte heparanase activities towards bovine endothelial ECM-bound HSPG were also shown to result in the formation of similarly sized HS digestion end products [3].

Incubation of extracts of the highly metastatic rat 13762 MAT and DMBA-8A cell lines and the non-metastatic variants J-clone and DMBA-Sask with HS resulted in complete degradation of the HS to 4.4 kDa fragments by the 13762 MAT and DMBA-8A heparanase activities, whereas the J-clone and DMBA-Sask extracts only partially degraded HS to fragments with number-average molecular masses of 8.1 and 12.5 kDa respectively (Figure 3). The highly metastatic 13762 MAT and DMBA-8A cell lines were previously shown to be more effective in their ability to degrade the artificial basement membrane Matrigel than the J-clone and DMBA-Sask variants in a basement-

Figure 3 Degradation of HS by homogenates of metastatic and non-metastatic rat mammary adenocarcinoma cell lines

HS was incubated at pH 5.1 for 16 h at 37 °C with no enzyme (■), homogenates of the non-metastatic variants DMBA-Sask (□) and 13762 MAT (J-clone) (▲) and the highly metastatic 13762 MAT and DMBA-8A cell lines (△) shown together for convenience. The incubation mixtures were fractionated by Superose 6 gel-filtration chromatography. Molecular-mass standards a–e are as described in Figure 1. For full details, see the Materials and methods section.

membrane-permeability assay [27], thus confirming the correlation of metastatic potential with heparanase activity [1].

Therefore porcine mucosal HS is proposed as a suitable natural substrate for the detection of heparanase activity in a variety of cell types, platelets and tissue extracts, and for assessing the heparanase content and the metastatic potential of tumour cell lines. However, because of the multiplicity of cleavage sites, the use of gel filtration to separate products from the undigested substrate makes it difficult to accurately quantify heparanase activity, particularly as multiple cleavages need to occur to allow separation of substrate from product. In addition, it is not known whether the relative rate for each of the cleavages is similar or whether the initial products of digestion may inhibit further cleavage by product inhibition, resulting in a lack of linearity of the assay with respect to time which has been observed for most of the HS-degrading exo-activities studied [45–48]. The lack of quantification of the heparanase activity combined with the time and labour required to analyse a single sample by gel-filtration analysis highlights the need for a rapid quantitative heparanase assay capable of detecting a minimum number of substrate cleavages.

Development of a quantitative heparanase assay

The interaction of heparin-binding proteins such as anti-thrombin III, acidic and basic fibroblast growth factor (FGF), platelet factor 4, β -thromboglobulin and HRG with HS has been well documented [49] along with more recent attempts to define their binding motifs on the HS molecule after chemical (primarily HNO_2 degradation) or bacterial heparinase and heparitinase digestion [5,6,42,50]. However, apart from the demonstration that mammalian heparanase can release active bFGF from the ECM [51], little work has been published on the interaction of HS-binding proteins with HS after mammalian heparanase degradation. While investigating the interaction of cHRG, an abundant plasma-derived HS-binding protein, with HS, we observed that not only did cHRG mask the mammalian MAT cell heparanase-cleavage sites on HS chains, but, after heparanase cleavage of HS, the cHRG-binding motif on HS was lost.

Figure 4 Degradation of HS as a function of incubation time and affinity of the digested HS products for cHRG-Sepharose

HS was incubated at pH 5.1 for 2 (□), 4 (△) and 16 (▲) h and in the absence of added enzyme for 4 h (■) at 37 °C. The incubation mixtures were applied to a cHRG-Sepharose column in PBS, washed with PBS and eluted by an NaCl gradient. The arrow indicates the start of the NaCl gradient. ———, Concentration of NaCl. For full details, see the Materials and methods section.

Therefore, after heparanase digestion, cleaved HS fragments bound less efficiently to cHRG-coupled Sepharose beads, enabling the development of an efficient and novel method of separating the HS substrate from the products of heparanase digestion.

When HS was applied to a cHRG-Sepharose column and eluted by a salt gradient at pH 7.2, the relatively weakly binding HS was eluted by 0.27 M NaCl (Figure 4) compared with lung heparin, which was eluted by 0.6 M NaCl (results not shown). However, the 4.3 kDa fragments after complete digestion of HS by a 13762 MAT cell homogenate did not bind to cHRG-Sepharose. When HS was then degraded by 13762 MAT cell heparanase using the standard assay at pH 5.1 for 2, 4 and 16 h, the assay mixture was divided into three and analysed by gel-filtration chromatography, binding to DEAE-Sepharose and binding to cHRG-Sepharose. Gel-filtration analysis yielded a similar profile to Figure 1 with products at 12.7, 8.0 and 4.4 kDa respectively. The application of the assay mixture, and the subsequent elution of radiolabelled HS products from the cHRG-Sepharose column by a salt gradient, demonstrated that the decrease in the number-average molecular masses of the HS fragments by gel filtration correlated with an increase in material not binding to cHRG-Sepharose (Figure 4), indicating that the HRG-binding motif on HS was lost after heparanase digestion. There was no apparent change in the affinity of the bound HS fragments towards the cHRG-Sepharose column over the period of the incubation. The binding of HS to cHRG-Sepharose (and the number-average molecular mass of HS during gel-filtration analysis) was not influenced by the assay blanks containing buffer only, 0.1 mg/ml BSA, heat-inactivated enzyme or active enzyme chromatographed with HS at 4 °C (results not shown). After application of the incubation mixtures to DEAE-Sepharose, all radioactivity remained bound in the presence of 0.3 M NaCl, indicating that the products of digestion were not due to desulphation by some endosulphatase activity [24,52,53].

To investigate whether the binding of HS to cHRG was masking a heparanase-sensitive cleavage site, HS was digested with 13762 MAT cell homogenate in the absence and presence of a 3:1 molar ratio of cHRG/HS (the 18.5 kDa HS substrate

Figure 5 Inhibition of HS degradation by cHRG

HS was incubated at pH 5.1 for 8 h at 37 °C in the absence of added enzyme or cHRG (■) and with rat 13762 MAT cell homogenate in the presence (△) and absence (▲) of a 3:1 (cHRG/HS) molar ratio of cHRG. The incubation mixtures were fractionated by Superose 6 gel-filtration chromatography. Molecular-mass standards a–e are as described in Figure 1. For full details, see the Materials and methods section.

appeared to be cleaved three times and each potential cleavage site may bind to a cHRG molecule). Figure 5 shows that, in the presence of cHRG, the rate of degradation of HS (resulting in the formation of the small 4.5 kDa fragments) was significantly retarded compared with cleavage in the absence of cHRG where the substrate was completely degraded.

Determination of heparanase activity

Using the cHRG–Sephacryl assay, maximal heparanase activity was observed at pH 5.1 (results not shown), similar to previous assays analysed by gel filtration (Figure 1). *N*-Acetylmannosamine was added to the incubation mixture to inhibit exoglycosidase release of [³H]GlcNAc which would not bind to cHRG–Sephacryl. The affinity of HS and the digestion products for cHRG–Sephacryl was not influenced by the incubation mixtures containing final concentrations of 0.1 mg/ml BSA, 0.1 % (v/v) Triton X-100 or up to 0.2 M NaCl. However, tissue homogenate heparanase activity was increased up to 2-fold when assayed in the presence of 0.1 % Triton X-100. In contrast, soluble heparanase activity (after removal of the membranes by centrifugation) was unaffected by the addition of BSA or Triton X-100 to the incubation mixture, although highly purified enzyme preparations required the presence of BSA in the incubation mixture for optimal activity and stability. The requirement of BSA for the determination of optimal activity of highly purified enzyme and Triton X-100 for maximal activity of tissue homogenates has been extensively reported for the determination of HS-degrading exoenzyme activities [45–48,54,55]. The cHRG–Sephacryl columns were routinely eluted with high-salt concentrations, and re-equilibrated in PBS. The columns were stored in PBS at 4 °C and were in constant use for over a 2-year period without appreciable decrease in binding capacity.

Figure 6 compares the change in apparent molecular mass after 13762 MAT homogenate heparanase digestion of HS (A), with the appearance of unbound radioactive HS fragments after application of the incubation mixture to cHRG–Sephacryl (B). Gel-filtration analysis of material not bound to cHRG–Sephacryl after a 6 h incubation showed an apparent molecular mass of 4.2 kDa (23 % of the initial size) and comprised 20 % of the applied radioactivity, whereas the bound material eluted by 1 M

Figure 6 Degradation of HS as a function of incubation time

HS was incubated with rat 13762 MAT cell homogenate at pH 5.1 for 2 (□), 4 (▲), 6 (△), 8 (●) and 10 (○) h and in the absence of added enzyme for 4 h (■) at 37 °C. The incubation mixtures were divided and fractionated by (A) Superose 6 gel-filtration chromatography and (B) binding to a cHRG–Sephacryl column where the unbound fraction was determined for each time point. Molecular-mass standards a–e are as described in Figure 1. For full details, see the Materials and methods section.

NaCl had an apparent molecular mass of 12.5 kDa (results not shown). Enzyme activity determined by cHRG–Sephacryl analysis was linear with respect to time for up to 10 h incubation and for up to 30 % conversion of substrate to product (Figure 6B). However, incubations that resulted in greater than 30 % conversion of substrate into product resulted in the loss of linearity of the assay with respect to time (results not shown), therefore the incubation period was modified to give less than 25 % conversion. After prolonged (24 h) incubation, 84 % of the radioactivity passed through the cHRG–Sephacryl column and had an apparent molecular mass of 4.2 kDa. Material remaining bound to the column presumably contained higher-affinity HRG-binding motifs or was heparanase-resistant, a phenomenon that was not investigated. Background material not binding to cHRG–Sephacryl was generally of low molecular mass, being less than 6 kDa, and may represent previously cleaved HS fragments obtained during isolation of the HS from the porcine mucosa. The fragments may be removed by prior affinity purification of the HS substrate using cHRG–Sephacryl, or by prior gel filtration to remove the lower-molecular-mass species.

hHRG–Sephacryl can be used satisfactorily in place of cHRG–Sephacryl for the determination of heparanase activity. However, the binding affinity of hHRG for Balb/c 3T3 cell surface HSPG was weaker than that observed for cHRG [56],

Table 1 Heparanase activity of metastatic and non-metastatic tumour cell lines

Tumour cell homogenate heparanase activity was assessed after fractionation of the incubation mixture on cHRG–Sepharose. Enzyme activity is expressed as mean $\pm$ S.E.M. for three separate experiments except where otherwise designated. For full details, see the Materials and methods section.

Tumour type	Cell line	Metastatic	Heparanase activity (pmol/h per 10^6 cells)
Rat mammary adenocarcinoma	13762 MAT	Yes	47.4 ± 2.2
	13762 MAT (J-clone)	No	12.8 ± 0.5
	DMBA-8A	Yes	35.6 ± 3.2
	DMBA-Sask	No	3.4 ± 0.1
Human colonic carcinoma	HCT 116	Yes	10.3, 10.4
Human monocyte	U937	No	7.6, 8.2
Human keratinocyte	KJD	No	7.8, 10.2
Murine Lewis lung carcinoma	D122	Yes	16.4 ± 1.1
	LLC	No	6.5 ± 0.5
Murine lung carcinoma	A3a	Yes	16.3 ± 1.2
	SP1	No	3.4 ± 0.2

and this was reflected by a higher affinity of cHRG–Sepharose for porcine mucosal HS than that observed for hHRG–Sepharose (results not shown). cHRG has the additional advantage that it can be purified in higher yield from plasma than hHRG (C. Parish, unpublished work). Commercially available bovine kidney HS can be used in place of porcine intestinal HS for the determination of heparanase activity. In contrast, commercial bovine intestinal HS cannot be used for the determination of heparanase activity as it has a molecular mass less than 5 kDa, and is essentially insensitive to mammalian heparanase cleavage according to both gel-filtration and cHRG–Sepharose analysis.

Analysis of heparanase activity in different biological samples

A variety of cell lines, tissues and platelet homogenates as well as serum from normal and cancer patients were tested for heparanase activity using the quantitative cHRG–Sepharose heparanase assay. Table 1 shows that the heparanase activity present in homogenates of the highly metastatic rat mammary adenocarcinoma 13762 MAT and DMBA-8A cell lines was 4- and 10-fold higher than for the respective non-metastatic J-clone and DMBA-Sask variants. These values closely correlate with both the relative ability of these cell lines to degrade an artificial (Matrigel) basement membrane [27] and for the cell homogenates to degrade HS as assessed by gel-filtration analysis (see above). The metastatic variants of the murine Lewis lung carcinoma (D122) and lung carcinoma (A3a) degraded HS 2.5- and 4.8-fold faster than their respective non-metastatic variants (Table 1). Previously, Nakajima et al. [7,8] had reported only a 1.5–2.2-fold difference in heparanase activity between extracts of the poorly metastatic B16-F1 subline compared with the more highly metastatic B16-BL6, -F10 and -B16b sublines using their solid-phase assay with de-N-sulphated re-N-acetylated HS.

The sensitivity of the heparanase assay described in this paper may be of use for the clinical assessment of cancer patients. Heparanase activity was determined in lung tumour tissue taken from six lung carcinoma patients. Of these, heparanase activity in the primary tumour of five patients was elevated from 2- to 4-fold compared with that from the patient's adjacent non-tumorous lung tissue (results not shown). Although heparanase activity was barely detectable in human plasma (less than

Table 2 Heparanase activity in human and rat cells and tissues

The range of heparanase activity in a variety of homogenates of human and rat tissue and cultured cells was assessed after fractionation of the incubation mixture on cHRG–Sepharose. For full details, see the Materials and methods section.

Cell or tissue	Heparanase activity (pmol/h per mg of protein)
Human platelets	2178–3591 ($n = 6$)
Human colonic carcinoma (HCT 116)	38.2–66.6 ($n = 4$)
HUVECs	21.0–42.6 ($n = 4$)
Human smooth-muscle cells	50.3, 75.9
Human lung fibroblasts	1.5, 3.0
Human keratinocyte (KJD)	62.2, 78.4
Rat 13762 MAT cells	141.1–349.2 ($n = 6$)
Rat liver	9.5–28.3 ($n = 6$)

4 pmol/h per ml of plasma), significant heparanase activity was detected in normal adult sera and in sera from patients with metastatic malignancies. Serum heparanase activity in the cancer patients tested was 2-fold higher than that observed for normal controls [229.7 ± 28.8 ($n = 8$) for cancer patients compared with 116.6 ± 13.3 ($n = 5$) pmol/h per ml of serum for controls], confirming the results observed for sera from malignant melanoma patients [9]. In fact, these authors observed a 4-fold increase in serum heparanase activity in patients where tissue metastases were present. Nakajima et al. [9,57] also observed that serum heparanase levels in rats were 17-fold higher than normal 30 days after injection of the highly metastatic 13762NF mammary adenocarcinoma cells, and enzyme activities in the sera of rats bearing MTLn3 tumours correlated well with the extent of the metastases.

Heparanase activity was also detected in a variety of human and rat cell lines, platelets and tissue homogenates (Table 2), which should represent valuable sources of the heparanase enzyme for future purification. For ease of comparison between cells and tissues, heparanase activity is expressed/mg of protein extracted. The development of this rapid and quantitative heparanase assay will be invaluable for the purification of heparanase activity from a variety of sources and will enable the rapid screening of a large number of putative heparanase inhibitors that we are currently developing.

General discussion

We have described a novel assay for the determination of heparanase activity in a variety of mammalian tissues, cells and bodily fluids. The assay has several advantages over conventional assays in (a) ease of preparation of relatively large quantities of a radiolabelled natural substrate while maintaining the native structure, (b) allowing the rapid and simultaneous determination of a large number of samples and (c) allowing accurate quantification of heparanase activity by detecting single-site cleavage of the substrate. The novelty of the assay lies in the use of the HS-binding protein HRG (which appears to protect the heparanase-cleavage sites of HS) to separate the uncleaved HS from the cleaved products. Thunberg et al. [30] had previously described the use of Sepharose-bound anti-thrombin III to separate the cleaved and uncleaved forms of an anti-thrombin III-binding octasaccharide substrate after digestion by human platelet heparanase. Although the binding motifs of HS recognized by several HS-binding proteins and growth factors have been described, the HS motif recognized by HRG is yet to be

determined. However, HRG has been shown to prevent the binding of anti-thrombin III to heparin [58] and FGF to cell surface HS [56].

Although mammalian heparanase activities are generally thought to be endoglucuronidase activities [59,60], the specific cleavage sites have yet to be determined. The human platelet enzyme has been shown to cleave within the anti-thrombin III-binding site between -GlcA-GlcNS3S- in a heparin-derived octasaccharide [30]. Preliminary studies indicate that the binding of cHRG to heparin-derived oligosaccharides is dependent on the size of the oligosaccharide and the degree of sulphation (C. Freeman and C. R. Parish, unpublished work). However, it is not known whether HRG binds to HS within a particular sulphated domain or spans adjacent sulphated domains perhaps overlapping a heparanase-sensitive site within or adjacent to the sulphated domains.

The protection of heparanase-sensitive areas of HS by HRG questions the role of HRG in plasma. HRG is the second most abundant heparin-binding protein in plasma next to anti-thrombin III, being found in sera of healthy human adults at approx. 125 µg/ml [61]. It contains a remarkably high content of histidine and proline residues (approx. 20% of the amino acid composition) and has been shown to exhibit a number of biological effects including the neutralization of the anti-coagulant activity of heparin [62] and the inhibition of the anti-proliferative effect of heparin on smooth-muscle cells [63]. HRG also interacts with metal ions [64], plasmin [65], thrombospondin [66], fibrinogen and fibrin [67] and plasminogen [68] where it reportedly stimulated its conversion into plasmin. More recently, HRG was shown to mask cell surface HS chains recognized by FGF-1 and -2 [56]. The authors suggested that HRG could displace FGF from the ECM making it available to responsive cells, or alternatively HRG could mask HS sequences on responsive cells thereby preventing FGF activation. Since mammalian heparanase can release FGF from HS in the ECM [51] and HRG can inhibit mammalian heparanase activities (this study), serum HRG (or that released during platelet degranulation) may not only inhibit the anti-coagulation activities of HS and heparin or prevent cleavage of endothelial cell surface HSPG. It may also act either to protect FGF binding/activation sites on HSPG at the initial time of tissue injury or to prevent heparanase-induced release of FGF from the ECM. The three-way interaction of HRG, FGF and heparanase with HS will be better understood when the respective HS-binding and -cleavage motifs for HRG and heparanase are determined and compared with that recognized by FGF.

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