Evidence that platelet and tumour heparanases are similar enzymes

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In order to enter tissues, blood-borne metastatic tumour cells and leucocytes need to extravasate through the vascular basal lamina (BL), a process which involves a battery of degradative enzymes. A key degradative enzyme is the endoglycosidase heparanase, which cleaves heparan sulphate (HS), an important structural component of the vascular BL. Previously, tumour-derived heparanase activity (which has been shown to be related to the metastatic potential of murine and human melanoma cell lines) was reported to cleave HS and be inhibited by heparin, as distinct from human platelet heparanase, which cleaved both substrates [Nakajima, Irimura and Nicolson (1988) *J. Cell Biochem.* **36**, 157–167]. We recently reported the purification of human platelet heparanase and showed that the enzyme is a 50-kDa endoglucuronidase [Freeman and Parish (1998) *Biochem. J.* **330**, 1341–1350]. We now report the purification and characterization of heparanase activity from highly metastatic rat 13762 MAT mammary adenocarcinoma and human HCT 116 colonic carcinoma cells and from rat liver using essentially the same procedure that was reported for purification of the human platelet enzyme. The rat 13762 MAT tumour enzyme, which has a native M_r of 45 kDa when analysed by gel-filtration chromatography and by SDS/PAGE, was observed to be an endoglucuronidase that degraded heparin and HS to fragments of the

same sizes as the human platelet enzyme does. N-deglycosylation of both the human platelet and rat 13762 MAT tumour enzymes gave, in each case, a 41-kDa band by SDS/PAGE analysis, demonstrating that the observed difference in M_r between the platelet and tumour enzymes may have been due largely to differences in the relative amounts of N-glycosylation. Two peptides were isolated following Endoproteinase Lys-C digestion of both the human platelet and rat 13762 MAT tumour heparanases and were shown to be highly similar. Both the rat liver and human colonic carcinoma heparanases also degraded both heparin and HS to fragments of the same sizes as the human platelet enzyme does. Western-blot analysis of an SDS/PAGE gel using antibodies raised against human platelet heparanase demonstrated that human platelet, human tumour and rat tumour heparanases were immunochemically cross-reactive. In conclusion, because of the similarities in their sizes, substrate specificities, peptide sequences and immunoreactivities, we propose that heparanase activities present in human platelets, rat liver and in rat and human tumour cells are, in fact, mediated by a similar enzyme.

Key words: cancer, ECM degradation, heparan sulphate, heparin, metastasis.

INTRODUCTION

Heparan sulphate (HS) is an important component of the extracellular matrix and the vasculature basal lamina, which functions as a barrier to the extravasation of metastatic and inflammatory cells [1,2]. In order to enter tissues, blood-borne metastatic tumour cells and leucocytes need to extravasate through the vascular basal lamina, a process that involves a battery of protease and carbohydrate-degradative enzymes, including the endoglycosidase heparanase, which cleaves HS [1,2]. Heparanase activity has been shown to be related to the metastatic potential of murine and human fibrosarcoma and melanoma cell lines [1,3–6], and of murine lung and rat mammary adenocarcinomas, and to be elevated in the serum of cancer patients and in the primary tumour compared with adjacent normal tissue [7]. We and others have also described heparanase activity in a number of human cell types and tissues, including platelets [8–11], placenta [12], skin fibroblasts [13], smooth-muscle cells [7], umbilical vein endothelial cells [7,14,15], macrophages [16], monocytes [17] and neutrophils [18], as well as rat liver [7,19], activated but not resting rat T-lymphocytes [20], rat ovarian granulosa cells [21] and normal and neoplastic murine B-lymphocytes [22].

The purification and characterization of several heparanase activities differing in size and substrate specificity have been reported. A 137-kDa endoglucuronidase activity, which cleaved

both heparin and HS, was purified from human platelets [11]. This contrasts with an 8-kDa endoglucosaminidase activity purified from human platelets that was associated with a family of CXC chemokines derived from the platelet basic protein, namely connective-tissue-activating peptide-III, neutrophil-activating peptide-2 and β -thromboglobulin (β TG) [9]. However, Pikas et al. [23] have shown subsequently that a commercially available β TG preparation cleaved HS by endoglucuronidase activity. Jin et al. [24] reported the purification of a 94-kDa tumour-derived endoglucuronidase activity from cultured murine melanoma B16 cells that cleaved HS but was inhibited by heparin [5]. However, subsequent immunoselection studies [25] and sequence comparison with the published N-terminal amino acid sequence [26] revealed that this protein was a member of the hsp90 family of stress proteins or molecular chaperones, and as such was unlikely to exhibit heparanase activity [27]. More recently, a 45-kDa HS-degrading heparanase that was inhibited by heparin was purified from human placenta [12,28].

A major impediment to studying the enzyme in detail has been the lack of a simple assay for heparanase activity. Using a rapid quantitative assay used for determining heparanase activity in human, rat and murine cell lines and tissues [7], we recently reported a new procedure for the purification of human platelet heparanase to homogeneity [8]. The enzyme was shown to be a 50-kDa endoglucuronidase that degraded both HS and heparin and was not immunochemically related to β TG. Ihrcke et al. [29]

Abbreviations used: HS, heparan sulphate; HUVEC, human umbilical vein endothelial cells; β TG, β -thromboglobulin.

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have confirmed that human platelet heparanase activity is distinct from β TG immunoreactive material. We now report the purification and characterization of the heparanase from the highly metastatic rat 13762 MAT mammary adenocarcinoma and metastatic human HCT 116 colonic carcinoma cell lines, from rat 13762 MAT tumour tissue and from rat liver using essentially the same procedure that was used to purify the human platelet enzyme [8]. Each of the tumour and liver enzymes exhibited a similar chromatographic behaviour and M_r as was reported for the platelet enzyme. The rat 13762 MAT tumour and human HCT 116 colonic carcinoma heparanases were demonstrated to be immunochemically related to the human platelet enzyme. In contrast to earlier studies [3–5], we observed that the tumour and liver enzymes degraded not only HS, but also porcine mucosal and bovine lung heparin, to the same size fragments as was observed for the platelet enzyme activity. We therefore propose that the heparanase activities present in platelets and tumour cells are in fact similar, and most likely identical.

MATERIALS AND METHODS

Cell lines and tissues

The following cell lines were cultured and harvested as described previously: rat 13762 MAT mammary adenocarcinoma and murine melanoma B16-F10 cells [30]; human colonic carcinoma HCT 116 cells [31]; and human umbilical vein endothelial cells (HUVECs) [14]. Cultured 13762 MAT cells (10^6 cells) were injected subcutaneously into the neck of Fischer F344 rats and the tumour (approx. 8 g in weight) excised 12 days later. Livers were excised from 10-week-old Fischer F344 rats. All cells and tissues were washed in normal saline and frozen at -80°C .

Heparanase assays

Radiolabelled porcine intestinal mucosal HS (ORG 553), porcine intestinal mucosal heparin and bovine lung heparin were prepared by de-N-acetylation with hydrazine and re-N-acetylation with [^3H]acetic anhydride [7]. [^3H]Heparin (0.7 Ci/mg) was obtained from NEN Life Sciences (Boston, MA, U.S.A.). Cell homogenates were prepared by suspending 5×10^6 cells (which had been previously washed in normal saline) in 0.2 ml of 0.1% (v/v) aqueous Triton X-100 and disrupting by freezing and thawing three times in a solid CO_2 /ethanol mixture.

Assay 1

Heparanase activity was determined towards radiolabelled HS and the products separated from the substrate using chicken histidine-rich-glycoprotein–Sephacrose beads [7]. Briefly, 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 to yield up to 20% breakdown of the substrate to products. Enzyme activity was expressed as pmol of product formed/h per mg of protein.

Assay 2

Heparanase activity towards 2 μg of radiolabelled HS or heparin was assayed as described above for 16 h at 37°C , and the product separated from the substrate by gel filtration of the incubation mixture on Superose 12 [7]. The chromatography columns were both equilibrated in and developed with 0.5 M NaCl at a flow rate of 0.25 ml/min with 0.5 ml fractions being collected directly into scintillation vials, to which 4 ml of scin-

tillation fluid was added, the radioactivity determined and the number average molecular mass calculated [32]. The columns were calibrated with *N*-[^3H]acetylglucosamine (221 Da) and radiolabelled heparin-derived M_r average standards (16.7, 10.6, 6.7 and 3.1 kDa) [7].

Purification of heparanase activities

Heparanase activities from cultured rat 13762 MAT cells, rat 13762 MAT tumour tissue, cultured human HCT 116 colonic carcinoma cells and rat liver were purified essentially by the same procedure as reported for the purification of human platelet heparanase [8], with modifications as described below. All procedures were performed at 4°C unless otherwise stated. Heparanase activity towards HS was determined using assay 1. Protein concentrations were estimated with a Bio-Rad Coomassie Brilliant Blue protein-assay kit (Bio-Rad, Richmond, CA, U.S.A.) using BSA as a standard. Human platelet heparanase was purified as described [8].

Briefly, separate suspensions of 50 g of rat 13762 MAT tumour tissue, 50 g of rat 13762 MAT cultured cells (approx. 5×10^9 cells) and 30 g of human HCT 116 colonic carcinoma cells (approx. 2×10^9 cells) were each freeze-thawed in 4 vols. (w/v) of 15 mM sodium dimethylglutarate buffer, pH 6.0, containing 0.5 M NaCl (buffer A), whereas 500 g of rat livers were homogenized in a blender and the homogenate centrifuged. The supernatants were applied to a Concanavalin A–Sephacrose column (1 ml of gel per 3 g of tumour tissue or washed cultured cells or per 5 g of rat liver) and heparanase activity eluted with α -methylmannoside. The eluate was applied to a zinc-chelating Sepharose column (1 ml of gel per 5 g of tumour cells or tissue, or per 10 g of rat liver) connected in series with a Blue A–agarose column (1 ml of gel per 15 g of tumour cells or tissue, or per 20 g of rat liver) equilibrated in buffer A. Following washing of the Blue A–agarose column with buffer B (60 mM Tris/HCl buffer, pH 7.2) containing 0.5 M NaCl, and with buffer B containing 0.8 M NaCl, the Blue A–agarose column was connected in series to an octyl-agarose column (1 ml of gel per 15 g of tumour cells or tissue, or per 50 g of rat liver). For purification of cultured rat 13762 MAT tumour-cell heparanase, the Blue A–agarose/octyl-agarose columns were also connected in series to a lentil-lectin–Sephacrose column (1 ml of gel per 25 g of tumour cells). The combined columns were eluted with buffer B containing 2 M NaCl; the eluate was concentrated, applied to two Superose 12 HW 10/30 columns connected in series and equilibrated in and developed with buffer A. For the purification of rat tumour-tissue heparanase, the Superose 12 eluate was further concentrated by Centricon 30 centrifugation to 0.2 ml, 1.8 ml of buffer C (20 mM Tris/HCl buffer, pH 7.2) was added and the solution concentrated to 0.2 ml again. The concentrated solution was diluted to 2 ml with buffer C and applied to a DEAE–Sephacrose column (1 ml of gel per 25 g of tumour tissue) equilibrated in buffer C. The column was washed with 5 vols. of buffer C, 4 M NaCl was added to the combined flow-through and wash to bring the NaCl concentration to 0.4 M and the solution was concentrated to 0.4 ml by Centricon 30 centrifugation.

Native and subunit M_r values

The native M_r values of the purified heparanase activities were determined by Superose 12 gel chromatography [8]. Discontinuous SDS/PAGE (10% gels) was carried out according to the method of Laemmli [33] and stained with Coomassie Brilliant Blue R250. Gels were calibrated with the following M_r standards: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor

(20 kDa) and α -lactalbumin (14 kDa). Samples (3 μ g) were reduced by boiling in dithiothreitol. Purified cultured rat 13762 MAT tumour and human platelet heparanase (10 μ g each) were de-N-glycosylated with N-glycosidase F as described previously [8] and analysed by discontinuous SDS/PAGE (10% gel), which was stained with Coomassie Brilliant Blue.

Purified human platelet heparanase (0.2 μ g), purified rat 13762 MAT tumour cell heparanase (3 μ g) and partially purified human HCT 116 colonic carcinoma heparanase (3 μ g) were each reduced, the proteins separated by SDS/PAGE (10% acrylamide) and transferred on to nitrocellulose, and Western-blot analysis performed using either rabbit anti-human platelet heparanase or pre-immune sera (1:10000 dilution) using the method described [8].

New Zealand white rabbits were immunized subcutaneously with 100 μ g of purified human platelet heparanase [8] emulsified with either complete Freund's adjuvant for the primary immunization, or incomplete Freund's adjuvant for subsequent boosting immunizations. Antibody titre was determined using an ELISA.

Partial amino acid sequence of human platelet and rat tumour heparanase

Human platelet and rat 13762 MAT tumour-cell heparanase were each digested *in situ* with Endoproteinase Lys-C following SDS/PAGE analysis of the purified enzymes. The peptides were recovered and the amino acid sequence determined as described in [34].

Determination of heparanase-susceptible linkages

Porcine mucosal HS, porcine mucosal heparin and bovine lung heparin (2 μ g each) were digested with purified cultured rat 13762 MAT tumour heparanase and the newly produced reducing terminals radiolabelled with [3 H]NaBH₄. Identification of the reducing-terminal sugars was determined as described previously [8].

RESULTS

Purification of mammalian heparanase

We have shown previously that the rat 13762 MAT mammary adenocarcinoma tumour cells, which are highly metastatic *in*

vivo, when grown *in vitro* are an extremely rich source of mammalian-tumour heparanase activity [7]. To obtain relatively large amounts of tumour-cell material more easily, cultured 13762 MAT tumour cells (10⁶ cells per rat) were injected subcutaneously into rats, which resulted in approx. 8 g of tumour tissue per animal being excised 12 days later. Extracts of the 13762 MAT tumour contained about the same heparanase specific activity as that reported for extracts of 13762 MAT tumour cells grown *in vitro* [7]. Tumours were discarded if they showed signs of necrosis or were bloody in appearance. Although the specific activity of heparanase in rat liver was relatively low, being less than a tenth of that found in 13762 MAT cell homogenates [7], rat liver was an abundant source of starting material. Tumour tissue, tumour cells and livers were frozen at -80°C for up to 6 months before use.

Heparanase was purified to apparent homogeneity from cultured rat 13762 MAT tumour cells, 13762 MAT tumour tissue and from rat liver by 3700-, 18200- and 102000-fold respectively (Table 1), using essentially the same procedure used for the purification of the human platelet enzyme [8]. Cultured rat 13762 MAT tumour cells (approx. 5×10^9 cells) and 13762 MAT tumour tissue (50 g of each), and 500 g of rat liver yielded 60, 36 and 120 μ g of purified heparanase respectively, with 8, 7 and 13% recovery of enzyme activity respectively from the original supernatants (Table 1). Human HCT 116 colonic carcinoma heparanase was purified partially to yield 20 μ g of protein with 10% recovery of enzyme activity from approx. 2×10^9 cells. SDS/PAGE analysis demonstrated the presence of several bands (see later). Subsequent attempts to further purify the relatively small amount of heparanase present resulted in loss of activity.

Approx. 75% of the total heparanase activity from each source was solubilized following homogenization by either repeated freeze-thawing of the rat 13762 MAT tumour tissue and 13762 MAT tumour cells or homogenization of the rat liver in a blender. The remaining membrane-associated enzyme could only be released by homogenization in buffer A containing 1% (v/v) CHAPS, but was not further characterized. All of the enzyme activity from each source, when applied to the Concanavalin A-Sepharose column, was bound tightly and eluted by α -methylmannoside (Table 1). Heparanase activity was purified by up to 220-fold with a usual recovery of 45–70% from the original supernatant (Table 1). As was observed during the purification of the human platelet enzyme [8], heparanase activity from each of the enzyme sources did not bind to the zinc-chelating Sepharose column, but was bound tightly to the Blue-A-agarose column,

Table 1 Purification of rat heparanase

Heparanase activities from cultured 13762 MAT cells, 13762 MAT tumour tissue and rat liver were purified essentially by the same procedure [8] with the addition of lentil-lectin-Sepharose(†) or DEAE-Sepharose(†) chromatography for purification of cultured 13762 MAT cell and 13762 MAT tumour heparanases respectively. n.d., not determined; –, DEAE-Sepharose chromatography not performed. Specific activity is given in nmol/h per mg of protein. For experimental details, see the Materials and methods section.

Step	Rat liver			Rat 13762 MAT tumour			Cultured rat 13762 MAT cells		
	Specific activity	Purification (-fold)	Yield (%)	Specific activity	Purification (-fold)	Yield (%)	Specific activity	Purification (-fold)	Yield (%)
Supernatant	0.005	1	100	0.305	1	100	0.403	1	100
Concanavalin A-Sepharose	1.11	220	68.4	3.85	25.1	47	21.4	53.5	
Combined Zn-Sepharose/ Blue-agarose/Octyl-agarose/ Lentil-lectin-agarose*	270.6	54125	20.5	814	2660	26	n.d.	n.d.	23.7
Superose 12	510.4	102000	13.0	1706	11150	18	1490	3752	8.2
DEAE-Sepharose†	–	–	–	2792	18242	6.6	–	–	–

Figure 1 Superose 12 chromatography of rat liver heparanase

Rat liver heparanase from purification step 4 was purified by gel-filtration chromatography on Superose 12. Fractions were assayed for heparanase activity (●) and monitored for protein (-----, A_{280}). Arrows 1 and 2 indicate V_E for BSA and ovalbumin respectively. For experimental details, see the Materials and methods section.

and heparanase activity in the 2 M NaCl eluate again did not bind to the octyl-agarose column (Table 1). Although it was bound tightly to Concanavalin A-Sepharose, cultured rat 13762 MAT cell heparanase did not bind to a lentil-lectin-Sepharose column and the passage of the Blue A eluate through the lentil-lectin column in series with the octyl-agarose column removed much contaminating protein. Heparanase activity from each of

the sources was purified by up to a further 240-fold following the combined dye matrix, and hydrophobic chromatography step, but with a loss of 50–66 % of the applied activity. No significant heparanase activity was detected in any of the buffer washes of the dye matrix column or remained bound to the hydrophobic column. In contrast to cultured rat 13762 MAT cell heparanase, approx. one-third of the heparanase activities from rat 13762 MAT tumour tissue and rat liver (as well as from human platelets) were observed to bind to a lentil-lectin column, suggesting a difference in glycosylation of the enzyme between tissue sources, although subsequent gel-filtration analysis indicated no difference in the apparent M_r between the respective lentil-lectin-bound and -unbound enzymes (results not shown).

Heparanase from both cultured rat 13762 MAT cells and from rat liver was finally purified by gel filtration (Table 1). A typical gel-filtration profile showing separation of heparanase activity from contaminating protein is shown for the purification of rat liver heparanase in Figure 1. Subsequent re-chromatography of the purified rat liver heparanase on Superose 12 resulted in a single coincident peak of heparanase activity and protein, albeit with a loss of at least half of the enzyme activity and protein (results not shown). Substantial losses of enzyme activity occurred following Centricon 30 concentration of each of the highly purified enzymes; however, the purified enzyme from each source was stable for over 2 months at 4 °C in buffer A at protein concentrations of 30 $\mu\text{g}/\text{ml}$. Rat 13762 MAT tumour heparanase required a further step for purification to homogeneity by passage through DEAE-Sepharose (Table 1), allowing a final separation of heparanase activity from the more acidic contaminating proteins.

Native and subunit M_r of heparanases from different sources

Gel-filtration analysis of heparanase purified from cultured rat 13762 MAT mammary adenocarcinoma and human HCT 116

Figure 2 SDS/PAGE of purified rat and human heparanases

Purified rat and human heparanase preparations were reduced with dithioerythritol and electrophoresed on a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue R250. (Left-hand panel) Lane 1, M_r standards; lane 2, cultured rat 13762 MAT cell heparanase; lane 3, rat liver heparanase; and lane 4, human platelet heparanase. (Right-hand panel) Lane 1, M_r standards; lanes 2 and 3, purified rat 13762 MAT tumour heparanase; and lanes 4 and 5, purified human platelet heparanase. Heparanases were incubated with either N-glycosidase F (lanes 2 and 4) or with no enzyme (lanes 3 and 5). For experimental details, see the Materials and methods section.

Figure 3 Western-blot analysis of purified rat and human heparanases

Purified rat and human heparanase was reduced with dithioerythritol and electrophoresed on 10% polyacrylamide gels. The gels were either (a) stained with Coomassie Brilliant Blue R250 or transferred on to nitrocellulose for Western-blot analysis performed using either (b) rabbit anti-human platelet heparanase or (c) pre-immune sera. Lane 1, human platelet heparanase; lane 2, human HCT 116 colonic carcinoma heparanase; and lane 3, cultured rat 13762 MAT cell heparanase. Arrows indicate the positions of the M_r standards of 94, 67, 43, 30, 20 and 14 kDa. For experimental details, see the Materials and methods section.

colonic carcinoma cells, from rat 13762 MAT tumours and from rat liver demonstrated that, in each case, the enzyme had an apparent M_r of 45 kDa (see Figure 1 for the rat liver heparanase gel-filtration profile). No enzyme activity was detected at M_r values corresponding to (i) 8–10 kDa or dimers or tetramers of the chemokine-sized platelet heparanase activity [9], (ii) 60 kDa, as reported for β TG-immunoreactive material in human prostate PC-3M cells [35], or (iii) 94 kDa, as reported for the B16 murine melanoma enzyme [24]. Extracts of cultured HUVECs, B16 murine melanoma cells, human colonic carcinoma HCT 116 cells, rat 13762 MAT mammary adenocarcinoma cells and rat liver also revealed an M_r of 45 kDa by gel-filtration analysis (results not shown).

SDS/PAGE analysis of heparanase purified from cultured 13762 MAT cells indicated a single Coomassie Brilliant Blue-staining band, corresponding to an M_r value of 45 kDa under both reducing (Figure 2, left-hand panel) and non-reducing conditions (results not shown), suggesting that the single-subunit protein has no significant intramolecular disulphide bridging as we observed for the 50-kDa human platelet enzyme [8]. The purified 13762 MAT tumour enzyme also had an apparent M_r of 45 kDa (Figure 2, left-hand panel), whereas the rat liver enzyme had a series of bands clustered between 43 and 47 kDa (Figure 2, left-hand panel). De-N-glycosylation of both the 50-kDa human platelet enzyme and the 45-kDa rat 13762 MAT tumour enzyme with N-glycosidase F resulted in a decrease in the apparent M_r in each case to 41 kDa by SDS/PAGE analysis (Figure 2, right-hand panel), demonstrating that the observed difference in M_r between the platelet and tumour enzymes may be due largely to differences in the relative amount of N-glycosylation.

Whereas the apparent native M_r of human HCT 116 colonic carcinoma heparanase was 45 kDa by gel-filtration analysis, SDS/PAGE analysis of the partially purified enzyme showed the presence of several Coomassie Brilliant Blue-staining bands (Figure 3a). Using rabbit polyclonal antibodies raised against the 50-kDa human platelet heparanase, Western-blot analysis of an SDS/PAGE gel of purified human platelet, purified rat MAT 13762 mammary adenocarcinoma cell and partially purified human HCT 116 colonic carcinoma heparanases demonstrated the presence of immunochemically cross-reactive species at M_r

values of 50, 45 and 50 kDa respectively (Figure 3b). Material was also stained at apparent M_r values of 67 and 70 kDa, which were also detected by pre-immune serum (Figure 3c).

Amino acid sequence of peptides from human platelet and rat tumour heparanases

Human platelet and cultured rat 13762 MAT tumour cell heparanases were each digested *in situ* with Endoproteinase Lys-C following SDS/PAGE analysis of the purified enzymes. Two peptides were recovered and amino acids sequenced from each of the enzyme digests. The two rat 13762 MAT tumour-derived peptides, LYGPDI (RM-1) and MVDEQT (RM-2), were highly similar to the respective human platelet-derived peptides LYGPDVGQPRRK (HP-1) and MVDDQT (HP-2), both of which are predicted within the human platelet cDNA sequence that we have isolated (C. Freeman and C. R. Parish, unpublished work). Searches of the dbEST (public EST, Genbank) database using Tfasta and the SWALL protein database using fasta3-t with each of the peptide sequences failed to find significantly similar sequences in any described protein containing either both rat (RM-1 and RM-2) or both human (HP-1 and HP-2) peptides. Gonzalez-Stawinski et al. [36] have recently published a partial amino acid sequence of a 55-kDa human platelet heparanase, which had been purified by an alternate procedure to our published method [8]. Peptide HP-1 that we have isolated is identical to their published Peptide 1, whereas HP-2 consists of the first six amino acids of their Peptide 2.

Substrate specificity of purified rat 13762 MAT heparanase

Heparanase activity towards porcine mucosal HS for each of the purified tumour and liver enzymes was maximal at pH 5.1, as determined by assay 1. Based upon gel-filtration analysis, porcine mucosal HS was cleaved in a stepwise fashion from 18.5 to 13 to 8 and finally to 4.5-kDa fragments by each of the purified enzymes (results not shown). The gel-filtration profiles were similar to our previous reports where purified human platelet heparanase and extracts of cultured rat 13762 MAT mammary adenocarcinoma cells cleaved HS in a stepwise fashion to 4.5-kDa fragments [7,8]. However, in contrast to previous reports for tumour heparanase activity towards heparin [3–5], rat liver heparanase and rat 13762 MAT mammary adenocarcinoma heparanase purified from both cultured cells and from tumours *in vivo* cleaved both porcine mucosal heparin and the more highly sulphated bovine lung heparin. Mucosal heparin was cleaved from 8.1 to 2.9-kDa fragments, whereas the more highly sulphated lung heparin was cleaved from 8.9 to 4.8-kDa fragments (Figures 4a and 4b), as we observed with the purified human platelet heparanase [8]. We also observed that extracts of rat liver, B16 murine melanoma cells, HUVECs, rat 13762 MAT mammary adenocarcinoma cells and human HCT116 colonic carcinoma cells cleaved both lung and mucosal heparin to similarly sized fragments (results not shown).

Previously, B16 murine melanoma cell heparanase was reported not to cleave porcine mucosal heparin [3–5]. However, we could clearly demonstrate cleavage of the substrate by utilizing a Superose 12 gel-filtration column, which enables superior resolution of the substrate from the product, and by the use of N-[3 H]acetylated heparin instead of heparin radiolabelled with [3 H]NaBH $_4$ at its reducing terminus. Commercially obtained heparin may have a large proportion of its reducing termini blocked by serine-linked peptides originating from the core protein. We observed that [3 H]NaBH $_4$ -reduced mucosal heparin had an apparent M_r smaller than that of N-[3 H]acetylated heparin and, in fact, close in size to the heparanase cleavage

Figure 4 Degradation of heparin by rat 13762 MAT tumour cell heparanase

(a) Porcine mucosal N-[^3H]acetylated heparin, (b) bovine lung N-[^3H]acetylated heparin and (c) reducing-terminal ^3H -radiolabelled porcine mucosal heparin were incubated with purified heparanase derived from cultured 13762 MAT tumour cells (●) or with no enzyme (○). The incubation mixtures were analysed by Superose 12 gel chromatography. Arrows indicate the peak elution of the 16.7, 10.6, 6.7, 3.1 and 0.2-kDa M_r standards. For experimental details, see the Materials and methods section.

product of N-[^3H]acetylated heparin (Figures 4a and 4c), indicating that the mucosal heparin chains able to be reductively radiolabelled were of small size and may already have been the

Figure 5 Phosphocellulose paper chromatography of acid-hydrolysed radiolabelled products following 13762 MAT tumour heparanase degradation of heparin

Following cultured 13762 MAT tumour cell heparanase digestion of porcine mucosal heparin, the products were reductively radiolabelled, hydrolysed by trifluoroacetic acid, nitrous acid and the acidic material following Dowex 1 chromatography, and separated by descending chromatography on phosphocellulose paper which was developed for 12 h in ethyl acetate/pyridine/5 mM boric acid (3:2:1). Arrows 1 and 2 indicate the positions of idonic and gulonic acids respectively. For experimental details, see the Materials and methods section.

result of prior heparanase cleavage. However, purified rat 13762 MAT mammary adenocarcinoma heparanase was demonstrated to cleave [^3H]NaBH $_4$ -reduced mucosal heparin (Figure 4c).

Identification of heparanase-susceptible linkages

HS chains generally consist of clusters of sulphated disaccharide units (predominantly N-sulphated glucosamine linked 1 $\rightarrow$ 4 to 2-sulphated α -L-iduronic acid residues) separated by long stretches of lowly or non-sulphated regions (predominantly disaccharide units of N-acetylated glucosamine linked 1 $\rightarrow$ 4 to β -D-glucuronic acid) [37,38]. Heparin is composed predominantly of sulphated disaccharide units (predominantly N,6-disulphated glucosamine linked 1 $\rightarrow$ 4 to 2-sulphated α -L-iduronic acid residues). To determine the residue that the tumour heparanase cleaves adjacent to, previously reduced HS, mucosal heparin and lung heparin were digested by purified cultured rat 13762 MAT tumour cell heparanase and the fragments radiolabelled by reduction of the newly produced reducing terminals. Dowex-1 chromatography of the acid-hydrolysed material revealed that 90 % of the radiolabelled material was acidic and that almost all of this co-ran with gulonic acid following phosphocellulose paper chromatography (Figure 5), thus confirming that the newly heparanase-generated reducing terminal in each case was a glucuronic acid.

DISCUSSION

We have now purified heparanase activities to apparent homogeneity from human platelets [8] and for the first time from rat liver, rat 13762 MAT mammary adenocarcinoma tissue and

cultured cells, and have partially purified heparanase from cultured human HCT 116 colonic carcinoma cells. There are five lines of evidence that suggest that the heparanase enzymes purified from each of these sources were similar if not identical. First, each of the enzymes was purified using essentially the same procedure that we reported for the purification of the human platelet enzyme [8]. Secondly, Western-blot analysis of an SDS/PAGE gel using antibodies raised against human platelet heparanase demonstrated that the human platelet, human tumour and rat tumour heparanases were immunochemically cross-reactive. Consistent with this observation was the fact that two human platelet heparanase-derived peptides, HP-1 and HP-2, were highly similar in their respective amino acid sequences to the peptides RM-1 and RM-2 respectively, derived from the rat 13762 MAT tumour heparanase.

Thirdly, each of the purified rat 13762 MAT mammary adenocarcinoma and rat liver enzymes, partially purified human HCT 116 colonic carcinoma enzyme, and extracts of cultured HUVECs, B16 murine melanoma cells, human colonic carcinoma HCT 116 cells, rat 13762 MAT mammary adenocarcinoma cells and rat liver have apparent M_r values of 45–50 kDa by gel filtration, SDS/PAGE or Western-blot analysis. These M_r values are consistent with the M_r values reported by us for the purified human platelet enzyme [8], and heparanase purified from human placenta [12,28], Chinese hamster ovary cells [39], partially purified from cultured human hepatoma cells [23] and human spleen [17] and extracts of murine macrophages [40]. De-N-glycosylation of both the 50-kDa human platelet enzyme and the 45-kDa rat 13762 MAT tumour enzyme with N-glycosidase F resulted in a decrease in the apparent M_r in each case to 41 kDa by SDS/PAGE analysis, demonstrating that the observed difference in M_r between the platelet and tumour enzymes may be due largely to differences in the relative amount of N-glycosylation. In fact, we have observed that human platelet heparanase cDNA encodes for a predicted core protein of 42 kDa with six putative N-glycosylation sites (C. Freeman and C. R. Parish, unpublished work). Several of the purified HS-degrading exo-enzyme activities isolated from human liver also were composed of several bands by PAGE, which were attributed to differences in peptide and glycosylation processing [41–43].

Fourthly, the purified human platelet, rat liver and rat tumour heparanases have similar substrate specificities. Purified rat 13762 MAT mammary adenocarcinoma heparanase cleaved N-[3 H]acetylated porcine mucosal HS to 4.5-kDa fragments, as was reported for purified human platelet heparanase [8] and by extracts of rat 13762 MAT mammary adenocarcinoma cells, HUVECs, human colonic carcinoma HCT 116 and KM 12-SM cells, B16-F10 murine melanoma cells and rat liver [7]. Previously, it was reported that B16 murine melanoma heparanase was unable to cleave reducing-terminal-radiolabelled [3 H]mucosal heparin [3–5]. However, in contrast, we have demonstrated that purified rat tumour and rat liver heparanase, and extracts of rat liver, B16 murine melanoma cells, HUVECs, rat 13762 MAT mammary adenocarcinoma cells and human HCT 116 colonic carcinoma cells will in fact degrade N-[3 H]acetylated porcine mucosal heparin and its more highly sulphated analogue, bovine lung heparin, to the same-sized fragments as found for the platelet enzyme [8]. Pikas et al. [23] have shown recently that human hepatoma and human platelet heparanase each cleaved both N-[3 H]acetylated porcine mucosal heparin and biosynthetically 35 S-labelled HS and had the same substrate specificities. By selecting high- M_r reducing-terminal-radiolabelled mucosal heparin as a substrate, B16 murine melanoma extracts were observed to degrade [3 H]heparin when cetylpyridium chloride precipitation was used to determine substrate

cleavage [40]. Similarly, using gel-filtration analysis, partially purified human placental heparanase was shown to degrade biosynthetically 35 S-labelled heparin at 40 % of the rate of HS degradation [44], whereas rat liver lysosomal heparanase was reported to cleave both N-[35 S]heparin and biosynthetically 35 S-labelled HS [45].

The rat 13762 MAT tumour enzyme was observed to degrade both heparin and HS through endoglucuronidase activity, as we reported for human platelet heparanase activity towards both heparin and HS [8]. These results are consistent with the endoglucuronidase activities others have reported for human platelets [11,46], B16 murine melanoma tumour heparanase [47], rat hepatocyte lysosomes [19], human placenta [44], Chinese hamster ovary cells [48], human hepatoma cells and a β -TG preparation isolated from human platelets [23]. Although HS consists of long stretches of GlcA-rich domains, it appears that only a small number of specific linkages are cleaved. A requirement for the presence of 2-O-sulphation for cleavage of HS by human platelet and Chinese hamster ovary cell heparanase has been reported, although differences were reported in the requirement for the presence of iduronic acid residues [23,48]. In contrast, we reported that the platelet enzyme had no absolute requirement for O-sulphation, provided the glucosamine residues were substituted [8]. To date, each of the studies has demonstrated only the absolute structural requirements for substrate cleavage to occur following prolonged incubation. A comparison of the influences of various substrate-structural features upon the rate of cleavage needs to be done to demonstrate important physiological structural components on both sides of the cleavage site.

Fifthly and finally, we have shown previously that human platelet heparanase activity towards HS is inhibited by the same heparin-derived analogues reported to inhibit B16 murine melanoma, murine lymphoma and rat tumour-derived heparanases [5,8,49–52]. Despite it being a heparanase substrate, heparin has also been reported to be a potent inhibitor of both platelet and tumour heparanase activities and of tumour metastasis [5,8,49–52]. However, although we have demonstrated that both the platelet and tumour enzymes do in fact cleave heparin, the resulting highly sulphated fragments are likely to also inhibit heparanase activity towards the less highly sulphated HS. For example, human platelet heparanase activity towards HS was inhibited by a 3.1-kDa heparin fragment [8], whereas B16 murine melanoma heparanase was potentially inhibited by heparin-derived tetradecasaccharides [51].

The heparanase activities that we have isolated in platelets and various cells and tissues may not be the only heparanase activities present in cells. Klein et al. [53] reported that stored HS in fibroblasts obtained from mucopolysaccharidoses patients (deficient in HS exo-degrading enzymes) was smaller than extracellular HS (indicating that endoglycosidase activity was functioning) but up to 80 % of the stored HS had reducing-terminal glucosamine residues, suggesting the existence of both endoglucuronidase and endoglucosaminidase activities. Bame and colleagues [39,48] have demonstrated that Chinese hamster ovary cells may contain a number of heparanase activities possessing different substrate specificities. Larsen et al. [54] found that the predominant materials in the plasma and urine following oral absorption of 35 S-radiolabelled HS and heparin by rats were di-, tetra- and hexa-saccharides. However, we have observed that HS is degraded to the same-sized fragments following prolonged incubation with both purified enzyme and with tissue and cell extracts and have found no evidence for the presence of other heparanase activities during our purification procedures ([7,8] and in this study). Therefore, there are unlikely to be other significant levels of heparanase activity apart from

the activities we have purified from the cell lines and tissues studied. In conclusion, based upon our observations of similar chromatographic behaviour during their purification, immunochemical cross-reaction and similarities in their peptide sequences, native and subunit M_r values, substrate specificities and inhibition of enzyme activity by modified heparins, we propose that the heparanase activities present in platelets, tumour cells and rat liver are in fact similar, and most likely identical, molecules.

Note added in proof (received 12 July 1999)

We [55] and Vlodavsky et al. [56] have recently shown that human platelet, human placental, mouse activated T-cell and rat tumour heparanases are highly similar in their amino acid and cDNA nucleotide sequences, further demonstrating that there is one dominant endoglucuronidase enzyme which is expressed by mammalian tissues, platelets and metastatic tumour cells.

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