Human mononuclear cells contain an endoglycosidase specific for heparan sulphate glycosaminoglycan demonstrable with the use of a specific solid-phase metabolically radiolabelled substrate

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Xyloside-initiated $^{35}\text{SO}_4^{2-}$ -labelled glycosaminoglycans were isolated from the medium of cultured bovine glomeruli and covalently coupled to Sepharose 4B to construct a solid-phase substrate suitable for the detection of endoglycosidases. The substrate is rendered specific for heparitinase by prior digestion with chondroitin sulphate ABC lyase and is insensitive to proteinase, neuraminidase and hyaluronidase. Normal human mononuclear cells are shown to contain a heparitinase. This enzyme appears to be cell-associated and can be partially purified from human spleen by heparin affinity chromatography.

INTRODUCTION

Heparan sulphate (HS) proteoglycans have been described as integral constituents of basement membranes and as plasma-membrane components in a variety of cell types [1]. The *N*-sulphate and *O*-sulphate residues of the carbohydrate chains confer a highly anionic character to the glycosaminoglycan (GAG) chain, and some residues may participate in specific functional recognition sequences, e.g. the glucosaminyl 3-*O*-sulphate in the heparin anti-coagulant pentasaccharide sequence [2]. Within basement membranes the anionic charge associated with HS GAG is important in controlling membrane permeability to charged macromolecules [3] and may also function in stabilizing interactions between laminin, nidogen and HS components of the membrane [4]. The strategic localization of HS GAG on cell surfaces has suggested a role for HS as a transmembrane mediator of cell–cell and cell–matrix interactions [5], involving fundamental cell functions such as cell adhesion [6] and cell growth [7].

Specific endoglycosidases able to degrade HS GAG may be important modulators of basement-membrane integrity and cell–cell and cell–matrix interactions and thus could influence basement-membrane permeability, cell adhesion and cell motility. HS-degrading enzymes have been described in tumour cells that show a propensity to metastasis. Melanoma [8] and lymphoma [9] cells secrete such an enzyme, the quantity of which correlates with the metastatic potential [10]. In man, a platelet-derived heparitinase has been described [11], and normal mouse macrophages [12] and rat T-lymphocytes [13] can be activated to express HS endoglycosidases. These cell types, platelets, macrophages and lymphocytes, are important participants in general inflammatory processes, e.g. wound healing, but the last two cell types also have specialized roles in immune-mediated responses. Rat spleen, without discrimination among cell

types including platelets, has been shown to contain an enzyme capable of hydrolysing heparin GAG [13a].

In order to obtain evidence for the role of HS-degrading enzymes in human autoimmune disease, we have investigated normal human mononuclear cells for the presence of this enzyme by using a metabolically labelled solid-phase HS substrate.

MATERIALS AND METHODS

Materials

Cell-culture media DMEM and RPMI 1640 and foetal-calf serum (FCS) were obtained from Northumbria Biochemicals, Cramlington, Northd., U.K. $\text{H}_2^{35}\text{SO}_4$ (carrier-free) was supplied by New England Nuclear. DEAE-Sepharose CL6B, QAE-Sephadex and Sephacryl S-400 were from Pharmacia. Lymphocyte separation medium and Mono-Poly separation medium were from Flow Laboratories. Optiphase MP scintillant and the TSK 2000 and 3000 h.p.l.c. columns were from LKB. Cell-medium supplements (tri-iodothyronine, insulin, cortisol, prostaglandin E_1) *p*-nitrophenyl β -D-xyloside, concanavalin A (Con A), and the enzymes HS lyase II (product no. H6512), chondroitin sulphate (CS) ABC lyase (C2905), trypsin, Pronase, neuraminidase and hyaluronidase, as well as CNBr and guanidinium chloride were from Sigma. Heparin was obtained from Calbiochem (catalogue no. 375095). All other chemicals were of AnalaR quality from BDH.

Methods

Production of ^{35}S -labelled GAG. Post-mortem bovine glomeruli were obtained within 2 h of death by standard methods [14] and cultured in DMEM with 5% FCS, 5 pM-tri-iodothyronine, insulin (5 $\mu\text{g}/\text{ml}$), penicillin (100 $\mu\text{g}/\text{ml}$), streptomycin sulphate (100 $\mu\text{g}/\text{ml}$), 50 nM-cortisol and prostaglandin E_1 (25 ng/ml) in an air/ CO_2 (19:1) atmosphere at 37 °C in 25 cm² Costar tissue-

Abbreviations used: GAG, glycosaminoglycan; HS, heparan sulphate; DMEM, Dulbecco-modified Eagle medium; FCS, foetal-calf serum; QAE-, quaternary aminoethyl; Con A, concanavalin A; PBS, phosphate-buffered saline (10 mM mixed sodium hydrogen phosphates/142 mM-NaCl, pH 7.4); MM, methyl α -mannoside; CS, chondroitin sulphate.

culture flasks. (We have not determined whether the hormones used are necessary for GAG biosynthesis.) When the primary outgrowth was confluent, the cells were incubated in labelling medium which consisted of DMEM without sulphate, 5% FCS, hormones as described above, streptomycin chloride (100 µg/ml), penicillin (100 µg/ml), 2 mM-*p*-nitrophenyl β-D-xyloside and 1 mCi of H₂³⁵SO₄/ml. After 3 days the cells were discarded and the medium was passed sequentially through a 7 mm × 50 mm DEAE-Sepharose CL6B ion-exchange column and then through a 6 mm × 300 mm QAE-Sephadex A25 column pre-equilibrated in labelling medium. This latter column delayed the unused ³⁵SO₄²⁻, which was then eluted in fresh labelling medium 30 ml later and was used to label a new batch of confluent glomerular cells.

Purification of ³⁵S-labelled GAG. The material from the culture medium that bound to the DEAE-Sepharose column equilibrated in 0.15 M-NaCl was eluted with a NaCl gradient in 10 mM-phosphate buffer, pH 7.0 (Fig. 1a). The 2 ml of radiolabelled material harvested from the DEAE-column was dialysed against 250 ml of 6 M-guanidinium chloride/10 mM-phosphate, pH 7.0, overnight and then chromatographed in this buffer on a Sephacryl S400 column (26 mm × 800 mm) (Fig. 1b). A single peak of labelled material was observed, the first third of which was discarded as it contained GAG bound to small polypeptides (results not shown).

Production of solid-phase ³⁵S-labelled GAG. A portion (100 ml) of the material isolated from the Sephacryl S-400 column was dialysed overnight against 1 litre of 100 mM-NaHCO₃, pH 8.7. Sodium dithionite was then added to 100 mM final concentration in order to reduce the nitro group of the xyloside to an amino group. After 30 min at 4 °C, the material was recovered by ion-exchange chromatography on DEAE-Sepharose as described above. The aminophenylxyloside GAG was then coupled to Sepharose 4B by the CNBr technique described by Cuatrecasas [15], with the modification that the coupling reaction with the activated Sepharose was done at pH 7.0 in phosphate-buffered saline (PBS); label equivalent to 100 µCi was allowed to couple to 10 ml of packed gel, resulting in approx. 30 µCi of bound label.

The solid-phase preparation was washed extensively in PBS and then digested sequentially with the following enzymes in 100 mM-Tris/HCl, containing 5 mM-CaCl₂, pH 7.0, for 2 h each: CS lyase, 100 units/ml; trypsin, 100 µg/ml; chymotrypsin, 100 µg/ml. The beads were then sequentially washed three times between each enzyme treatment. The beads were next incubated for 1 h at 20 °C with 1 mM-tosylphenylalanylchloromethane ('TPCK') and 1 mM-tosyl-lysylchloromethane ('TLCK'). They were then incubated with 1 mg of heparin/ml for 2 h at 37 °C and finally washed with 3 M-NaCl in PBS, followed by PBS alone. The beads so prepared were snap-frozen in liquid N₂ and stored until use. Another aliquot of labelled beads was treated similarly, with the exception that flavobacterial HS lyase (10 units/ml) was used instead of CS lyase.

Solid-phase assay for HS-degrading enzymes. Beads containing approx. 2000 c.p.m. of labelled material were suspended in 100 µl of 100 mM-Tris/HCl buffer, pH 7,

containing 1.5 mM-CaCl₂ and 1 mM-MgCl₂ (for human-derived samples) or 5 mM-CaCl₂ (for flavobacterial HS lyase- or CS lyase-containing samples). A 300 µl portion of the test sample was added, diluted if necessary in the same buffer and, after mixing, the tube was incubated at 37 °C for 2 h without agitation (in order to promote interactions between cells and beads, both were allowed to co-sediment). The tube was re-mixed and the beads removed by centrifugation at 3000 *g* for 2 min. Half the supernatant was counted for radioactivity with LKB Optiphase MP scintillant and a Rackbeta 2 counter, and the results were expressed as percentage of the radioactivity on the gel released into the whole supernatant.

To measure heparitinase-like activity in the sample, we used the beads that had been digested with CS lyase. In addition to using a control with buffer alone, further controls were run with beads pre-treated with HS lyase, which would be expected to release their label in the presence of chondroitinase.

Release of label from the substrate pre-treated with CS lyase was determined by using various concentrations of flavobacterial HS lyase from 1 unit/ml to 100 units/ml. The beads previously digested with HS lyase were tested with CS lyase (1 unit/ml to 10 units/ml). Both sets of beads were tested with up to 1 mg of Pronase, hyaluronidase or neuraminidase/ml.

Units of activity adopted for CS lyase (and flavobacterial HS lyase) are as follows: 1 unit of enzyme is that quantity which, at pH 7, in the presence of 5 mM-Ca²⁺ at 37 °C, will cleave 1 nmol of endoglycosidic bonds in CS type A (or HS)/min as measured spectrophotometrically at 232 nm.

When comparisons between two different test substances were made, both were (if necessary) diluted until (a) release by both was less than 5% of label on the solid phase, and (b) release by the more active sample was less than 1.4 times the release by the less active sample. The relative activity of the two diluted samples was then adjusted by the corresponding dilutional factors to obtain the calculated relative activity of the original undiluted samples. This was done in order to avoid non-linearities of assay response with more active samples.

Preparation and culture of normal human mononuclear cells. Blood from healthy laboratory workers, given with their informed consent, was centrifuged over Lymphoprep (Flow Laboratories) in the standard manner. The cells remaining above the Lymphoprep were washed three times in Hanks solution without Ca²⁺ or Mg²⁺, centrifugation being at 500 *g* for 5 min. Platelets remaining in the supernatant were themselves washed, centrifugation being at 14000 *g* for 2 min. Mononuclear cells and platelets were counted. The mononuclear-cell fraction still contained residual platelets. It was therefore compared (for control purposes) with the platelet fraction (which contained virtually no mononuclear cells) diluted to the same concentration as the platelets in the mononuclear cell fraction.

In a similar manner a polymorph fraction was produced using Mono-Poly medium, and it was compared with a polymorph-free control containing the same concentration of platelets as in the polymorph fraction.

The mononuclear-cell and platelet fractions were assayed fresh (at 10⁷ mononuclear cells/ml and the corresponding concentration of platelets), after one cycle of freeze-thaw lysis, and both lysed and unlysed after 1, 2,

4, 5 and 8 days in culture. Culture was in RPMI 1640 medium (Dutch modification) with 10% FCS, and 0, 1, 10 or 20 μg of Con A/ml, at a cell concentration of $1 \times 10^6/\text{ml}$, in 96-well Costar polystyrene round-bottomed tissue-culture plates. After culture, cells were transferred by using polyethylene pipettes to a 10 ml centrifuge tube and re-concentrated by centrifugation at 1000 g for 5 min. Subsequent assay was at a cell concentration corresponding to $10^7/\text{ml}$ of cells going into culture. Moreover, after a 4-day culture without Con A, a freeze-thaw lysate of cells was assayed with and without freshly added Con A (25 $\mu\text{g}/\text{ml}$). Activity in the sample with Con A was markedly lower, but activity could be restored by the addition of 15 mM-methyl α -mannoside (M α M). 15 mM-M α M by itself caused no label release, nor did it enhance release in the absence of Con A. All cultures that had contained Con A were therefore assayed in the presence of 15 mM-M α M.

Labelled material released from the solid phase by 4-day-cultured mononuclear cells without Con A was chromatographed on a TSK 2000 600 mm $\times$ 7.5 mm h.p.l.c. column, as was material released from the solid-phase by flavobacterial HS lyase.

Partial purification of HS endoglycosidase from human spleen. Human peripheral blood provided an inadequate number of mononuclear cells for preparative purposes. We therefore chose to use fresh post-mortem human spleen as a source of mononuclear cells. Although we accept that the starting cell population may be different from that found in peripheral blood, we are forced to make the assumption that the expressed enzyme will be qualitatively the same. A whole fresh post-mortem human spleen was pressed through a 250 μm sieve to achieve a single-cell suspension. Contamination with cellular fragments and platelets was diminished as far as possible by repeated centrifugation at 500 g and discarding of the supernatant. The cells were then freeze-thaw-lysed in a volume of PBS equal to the packed cell volume, supplemented with 10 mM-*N*-ethylmaleimide, 10 mM-benzamide, 1 mM-phenylmethanesulphonyl fluoride and 10 mM-EDTA, pH 7. The lysate was chromatographed on a 26 mm $\times$ 750 mm heparin-Sepharose 2B affinity column and eluted with 500 ml of an NaCl gradient (150 mM–1.6 M). Those fractions showing greatest activity were pooled, concentrated, then chromatographed in 500 mM-NaCl/10 mM-phosphate, pH 7, on a 600 mm $\times$ 7.5 mm TSK 3000 h.p.l.c. column, to determine the molecular mass of the enzyme.

RESULTS

Production of xyloside-initiated solid-phase GAG

Xyloside-treated glomerular cells produced sulphated GAG, which could be harvested from the culture medium by ion-exchange chromatography (Fig. 1a).

Gel filtration of culture medium (Fig. 1b) showed a significant shift from two peaks of [^{35}S]GAG of K_{av} 0.21 and 0.47 in the untreated medium to a single peak of K_{av} 0.78 in the xyloside-treated medium.

After reduction of the nitrophenyl xyloside to the amino form with dithionite, it was possible to couple approx. 60–70% of the $^{35}\text{SO}_4^{2-}$ label to the solid-phase matrix. Analysis of the purified [^{35}S]GAG after attachment to Sepharose showed a CS/HS ratio of 4:1 as assessed by sensitivity to chondroitinase and heparitinase.

Fig. 1. (a) Elution of ^{35}S -labelled material produced by bovine glomerular cells from DEAE-Sepharose with an NaCl gradient and (b) gel-filtration chromatography of material eluted from (a) in 6 M-guanidinium chloride on Sephacryl S-400

(a) ^{35}S -labelled bovine-glomerular-cell nitrophenyl xyloside-initiated culture medium was applied to a 7 mm $\times$ 50 mm DEAE-Sepharose column at 10 ml/h. After the column had been washed with 100 ml of PBS, a gradient of NaCl in PBS (....) was applied. Fractions were collected and counted for radioactivity (—). (b) Pooled labelled material eluted from (a) above, dialysed against 6 M-guanidinium chloride/10 mM-phosphate, pH 7, was chromatographed in this buffer on a 26 mm $\times$ 800 mm Sephacryl S-400 column at 30 ml/h, and fractions were collected and counted for radioactivity (Δ). A similar experiment using non-nitrophenyl xyloside-initiated medium was conducted for comparison ($\bullet$).

This solid-phase GAG substrate could be rendered specific for HS-degrading enzymes by extensive digestion with CS lyase or made specific for chondroitinases by prior digestion with flavobacterial heparitinase. The sensitivity and specificity of assays using solid-phase substrates prepared in this way is shown in Figs. 2 and 3. By using the heparitinase-sensitive substrate, it was possible to detect 10 munits of flavobacterial heparitinase/ml, which released more label from the solid phase than 10 units of chondroitinase ABC/ml, or 1 mg of Pronase, hyaluronidase or neuraminidase/ml. About 95% of $^{35}\text{SO}_4^{2-}$ -labelled GAG could be released by 100 units of flavobacterial heparan sulphate lyase/ml. By using the chondroitinase-sensitive substrate, it was possible to detect 200 munits of CS lyase, and 90% of the ^{35}S could be released by 10 units of enzyme/ml. HS lyase (10 units/ml) and Pronase (1 mg/ml) released less than 3% of the radioactivity on the solid phase.

Fig. 2. Release of ³⁵S-labelled fragments from heparitinase-sensitive substrate (i.e. substrate pre-treated with CS lyase)

A portion (≈ 2000 c.p.m.) of CS lyase-pretreated [³⁵S]-GAG-Sepharose beads was incubated for 2 h at 37 °C with HS lyase, CS lyase, Pronase or buffer only (control). Half the supernatant in each tube was counted for radioactivity, and the total percentage of label released was calculated.

Fig. 3. Release of ³⁵S-labelled fragments from HS chondroitinase-sensitive substrate (i.e. substrate pretreated with HS lyase)

A portion (≈ 2000 c.p.m.) of HS lyase-pretreated, [³⁵S]GAG-Sepharose beads was incubated for 2 h at 37 °C with HS lyase, CS lyase, Pronase or buffer only (control). Half the supernatant in each tube was counted for radioactivity, and the total percentage of label released was calculated.

Detection of mononuclear-cell heparitinase

Mononuclear cells, i.e. lymphocytes and monocytes, and polymorphonuclear cells, were assayed for the presence of HS-degrading enzymes. Fig. 4 shows the release of label by freeze-thaw-lysed mononuclear cells and platelet controls from the heparitinase-sensitive substrate using cells freshly isolated and after various times in culture. In a separate experiment to assess reproducibility, ten replicates of 3×10^7 cells/ml from a single individual gave a release of 5.3 ± 0.34 % (mean $\pm$ S.D.). Freshly isolated mononuclear cells consistently showed twice the degradative capacity of the platelet control. After culture, the amount of release in the platelet control fell, whereas that in the mononuclear-cell fraction increased approx. 2-fold after 4 days in culture. Lysed mononuclear cells from five subjects after 4 days in culture showed at least a 4-fold increase over the platelet control for each subject (results not shown). Such a comparison must of course be interpreted with the reservation that the access to the solid-phase substrate of intact cells and of solubilized cell contents may be very different.

Fig. 4. Label release from heparitinase-sensitive substrate by cultured normal human peripheral-blood mononuclear cells with platelet contamination and by appropriate controls

A 300 µl portion of peripheral-blood mononuclear cells (10^7 /ml) contaminated with platelets (2×10^7 /ml) (■) or 300 µl of platelets (2×10^7 /ml) alone (△), were incubated after various times in culture with CS lyase-pretreated [³⁵S]GAG beads for 2 h at 37 °C; half the supernatant was counted for radioactivity and the total percentage of label released was calculated. ○, Culture supernatant.

Fig. 5. Gel-filtration h.p.l.c. of [³⁵S]fragments released from heparitinase-sensitive substrate by flavobacterial lyase (—) or by mononuclear-cell heparitinase (·····)

A 20000 c.p.m. portion of CS lyase-pretreated [³⁵S]GAG beads was incubated with 4-day-cultured peripheral-blood mononuclear cells (10^7 /ml) or with flavobacterial HS-lyase (100 munits/ml) for 2 h at 37 °C. The supernatant was fractionated in 100 mM-phosphate, pH7, on a TSK 3000 600 mm $\times$ 7.5 mm column at 0.5 ml/min. Abbreviations: EG, ethylene glycol; PEG, poly(ethylene glycol); vv, void volume; tbv, total buffer volume.

Stimulation of mononuclear cells with various doses of Con A had no effect on the expression of heparitinase activity.

When freshly isolated or cultured lysed or unlysed mononuclear cells were incubated with the chondroitin-

Fig. 6. (a) Elution of mononuclear-cell heparitinase activity (—) from heparin-Sepharose column with an NaCl gradient (.....) and (b) gel-filtration h.p.l.c. of spleen cell-heparitinase

A portion (100 ml) of freeze-thaw spleen lysate with proteinase inhibitors was applied in PBS to a 26 mm $\times$ 750 mm heparin-Sepharose 2B column at 0.5 ml/min. After the column had been washed with PBS, a gradient of NaCl in PBS was used to elute bound material. Fractions were tested for heparitinase by the assay described in the text. (b) Partially purified spleen heparitinase was chromatographed in 500 mM-NaCl/10 mM-phosphate, pH 7, on a TSK 3000 7.5 mm $\times$ 600 mm column at 0.5 ml/min. Fractions were assayed for heparitinases by the assay described in the text. Various proteins were used as M_r markers.

Fig. 7. pH profile of mononuclear-cell heparitinase and buffer-only controls

Aliquots of partially purified spleen enzyme (Δ) or buffer-only control ($\bullet$) were assayed for heparitinase activity in Ca^{2+} -containing buffers at pH 2–10 using CS lyase-pretreated [^{35}S]GAG beads.

ase-sensitive substrate, no release of label above the buffer-only control was observed. Supernatant from cells cultured for various times was tested on both the heparitinase-sensitive and the chondroitinase-sensitive substrate and contained no detectable activity. Mononuclear cells were subjected to freeze-thaw lysis, either freshly isolated or after culture, and there was an increase in heparitinase activity, which varied from person to person, between 2- and 8-fold (results not shown) (results for freeze-thaw-lysed cells is shown in Fig. 4).

To determine the size of fragments released from the solid-phase HS substrate by 4-day-cultured lysed mononuclear cells, the material was analysed by h.p.l.c. on a TSK 2000 column (Fig. 5). The fragments released by the mononuclear cells were heterogeneous in size and of higher molecular mass than that released by flavobacterial HS lyase, with the peak corresponding to material of M_r 3000 on a column calibrated with poly(ethylene glycol) molecular-mass standards.

Partial characterization of heparitinase from spleen-cell suspension

The heparitinase activity in the freeze-thaw extract of human spleen was bound to the heparin affinity column at physiological ionic strength and could be eluted from the column by using 0.6–0.7 M-NaCl (Fig. 6a). The pooled fractions from this column were applied to the TSK 3000 h.p.l.c. column and the heparitinase activity was eluted as a single peak equivalent to a component of 50 kDa (Fig. 6b). The spleen-derived enzyme was incubated with solid-phase HS substrate at 37 °C in 0.15 M-NaCl containing 150 μg of bovine serum albumin/ml in buffers of pH 2–10. The release of HS GAG from the solid phase showed a significant pH-dependence consistent with an enzyme-mediated reaction with a broad pH optimum from pH 4.0 to 7.0 (Fig. 7).

DISCUSSION

We have identified, in normal human mononuclear cells, an endoglycosidase specific for HS GAG, using a metabolically labelled [^{35}S]HS substrate, initiated on nitrophenyl xyloside and covalently bound to a solid-phase matrix. CS and HS chains are normally initiated on a β -xylose moiety linked to a core-protein serine residue [16], and xylosides have been used as artificial initiators of both CS [16] and HS [17] GAG. Xylosides have been shown to cause a several-fold stimulation of free CS chains by a variety of cell types [18–21], but they are less effective at initiating synthesis of free HS chains and the effect is very dependent on cell type [16,21,22].

We have found that early-passage bovine glomerular cells secrete predominantly CS GAG into the culture medium in the presence of nitrophenyl xyloside. Analysis of this purified [^{35}S]GAG after attachment to a solid-phase matrix showed a CS/HS ratio of 4:1, as assessed by sensitivity to CS lyase and HS lyase. Extensive digestion of the solid-phase GAG preparation with CS lyase produced a substrate insensitive to proteinases, which was > 95% degradable by bacterial HS lyase. By employing [^{35}S]GAG initiated on nitrophenyl xyloside, we were able to couple GAG to a solid-phase matrix very efficiently by following a simple reduction procedure. The link proved to be stable, with spontaneous leakage of $^{35}\text{SO}_4^{2-}$ of less than 1% and sensitivity to protease was

minimal (< 2%), unlike substrate prepared with purified native HS proteoglycan (results not shown).

Previously, heparitinase activity has been detected by using ^{125}I -labelled chemically modified heparin [11,23], covalently bound to Sepharose 4B, or alternatively by chromatographic analysis of the ^{35}S released from extracellular matrix coated on plastic dishes [24]. In general, published assays for heparitinases lack detail as to assay sensitivity and specificity, and although Nakajima *et al.* [23] reported 82% release of the ^{125}I -labelled modified heparin by 500 units of enzyme/ml, they did not report release values with smaller quantities of enzyme. Most other heparitinase assays fail to report sensitivity to bacterial HS lyase at all [11,24]. The use of heparin instead of HS as a substrate may detect enzymes with a preference for highly sulphated sequences which are under-represented in HS and may detect inefficiently enzymes with a specificity for *N*-acetylated moieties, which are less common in heparin (80% of glucosamine residues are *N*-sulphated [25]). The assay for heparitinases employing chromatographic analysis of enzyme-solubilized $^{35}\text{SO}_4^{2-}$ from extracellular matrix labelled *in vitro* also has disadvantages: the method is time-consuming, involving a 24 h incubation with substrate, followed by gel filtration for each sample [24]. Furthermore, this assay is sensitive to proteolytic degradation, which has been reported as a prerequisite for optimum detection of heparitinase activity from ESb lymphoma cells [26].

The endoglycosidase activity that we have detected in mononuclear cells is specific for HS GAG and does not degrade a CS GAG substrate prepared in a similar way, but with prior heparitinase treatment. Thus the agarose-xyloside link is not being cleaved. Furthermore, the size of the degradation products on h.p.l.c. analysis (Fig. 5) eliminated the involvement of sulphatase or exoglycosidases.

The sequence specificity of the endoglycosidase that we have detected has not been determined. However, the HS-degrading enzymes from melanoma [10] and human platelets [11] were identified as endo- β -D-glucuronidases that cleave β -D-glucuronosyl-*N*-acetylglucosaminyl linkages. Although these enzymes show a similar specificity, they differ in molecular mass (134 kDa for the platelet enzyme and 96 kDa for the melanoma enzyme).

HS-degrading activity was associated with viable mononuclear cells, but was not detected in cell-culture medium and therefore is not a secreted enzyme under the test conditions. Culture of mononuclear cells for 4 days produced an increase in cell-associated enzyme, but proliferating cells stimulated by Con A showed no further increase in enzyme activity. As freeze-thawed cell extracts showed increased activity compared with intact cells, the location of the enzyme was not restricted to the outside of the plasma membrane. Interestingly, rat T-lymphocytes have been shown to contain an HS endoglycosidase which is present in cell lysates of normal cells in either unstimulated or activated states [27]. Secretion of this latter enzyme into culture medium could be induced by stimulation of resting cells with soluble specific antigen. In murine macrophages [12], only activated cells express HS-degrading activity, which is cell-associated and is not secreted. Our preliminary experiments to identify HS endoglycosidase within purified T- and B-lymphocytes and monocytes suggests that the enzyme activity is associated with all three cell types (R. F. Sewell, P. E. C. Brenchley & N. P. Mallick, unpublished work).

As others have suggested [27], the demonstration of HS endoglycosidase in mononuclear cells may reflect a role for the enzyme in controlling the normal migration of these recirculating cells from one compartment to another (i.e. from blood vessels through basement membrane to extravascular tissue, or from blood to lymph through extracellular matrix of endothelial cells, forming high endothelial-cell venules). However, inappropriate expression of this enzyme could potentially also damage the HS-rich vascular endothelial-cell glycocalyx or even alter the HS component within basement membranes such as the glomerular basement membrane. Mononuclear cells may have direct access from the glomerular capillaries through the fenestrated endothelium to the glomerular basement membrane, which if reduced in anionic HS GAG could lead to the loss of charge-selective filtration associated with renal lesions such as minimal-change nephropathy. Elucidation of the mechanisms regulating expression of HS-degrading enzymes in mononuclear cells should help in our understanding of the normal and pathological roles of HS GAG.

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