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Differential Partition of Anticoagulant Heparan Sulfate Proteoglycans Synthesized by Endothelial and Fibroblastic Cell Lines

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Abstract The heparan sulfate proteoglycans that bind and activate antithrombin III (aHSPGs) are synthesized by endothelial cells as well as other nonvascular cells. We determined the amounts of cell surface–associated and soluble aHSPGs generated by the rat fat pad endothelial (RFP) cell line and the fibroblast (LTA) cell line. The RFP cells exhibit higher levels of cell surface–associated aHSPGs as compared to LTA cells, whereas LTA cells release larger amounts of soluble aHSPGs as compared to RFP cells. After confluence RFP cells show an increase in both cell surface–associated and soluble aHSPGs. In contrast, postconfluent LTA cells maintain a constant level of cell surface–associated and soluble aHSPGs. These observations indicate that different cell types can preferentially accumulate aHSPGs as cell surface–associated or soluble forms which could reflect alternate biological functions.

Key words: antithrombin binding, extracellular matrix, glycosaminoglycans, heparan sulfate proteoglycans, anticoagulant heparan sulfates, ligand binding assay

Endothelial cells synthesize a subset of heparan sulfate proteoglycans (aHSPGs) that bind and activate antithrombin III (AT). These species accumulate in the extracellular matrix (ECM) of cultured microvascular endothelial cells and on the subendothelial basement membrane of rat aorta [de Agostini et al., 1990a]. The current evidence suggests that expression of aHSPGs is not restricted to endothelial cells, but also occurs with fibroblasts [Marcum et al., 1986] and takes place in close proximity to Reichert's membrane [Pejler et al., 1987].

The rat microvascular endothelial cell (RFP) as well as the mouse fibroblast (LTA) lines synthesize aHSPGs. RFP cells also accumulate aHSPGs after confluence in the surrounding ECM [de Agostini et al., 1990a,b]. The HSPGs from RFP cells have recently been isolated and cloned, revealing the presence of the proteoglycans ryudocan and the rat analog of syndecan [Kojima et al., 1992a,b]. The two cDNAs encode for integral membrane proteins, which indicates that aHSPGs are initially exposed on the cell surface as membrane intercalated proteoglycans and are then released either to be bound on ECM or remain in soluble form.

In the current study we determined the partition of RFP and LTA cell aHSPGs between a cell surface form (intercalated in the plasma membrane or bound to ECM) and a soluble form released into the culture media. This investigation required the development of a sensitive ligand binding assay to quantitate soluble aHSPGs and the use of a previously described cell binding assay to measure cell surface–associated aHSPGs [de Agostini et al., 1990a].

METHODS

Materials

Purified human AT was purchased from Cutter Biological (Berkeley, CA). *Flavobacterium*

Abbreviations: AT, antithrombin; BSA, bovine serum albumin; ECM, extracellular matrix; DMEM, Dulbecco's minimum essential medium; HSPGs, heparan sulfate proteoglycans; aHSPGs, antithrombin binding fraction of HSPGs; iHSPGs, AT binding depleted fraction of HSPGs; LTA, subline of mouse fibroblastic L-cells; PBS, phosphate-buffered saline; PMSF, phenylmethane sulfonylfluoride; RFP, rat fat pad microvascular endothelial cells.

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heparitinase was purified as previously described [Marcum and Rosenberg, 1984]. Chondroitinase ABC and dextran sulfate (M_r 5,000) were obtained from Sigma (St. Louis, MO). Blue dextran 2,000, dextran T70, Sephadex G-25, G-50, and G-75, and Sepharose C14B were purchased from Pharmacia (Uppsala, Sweden). Sodium heparin from porcine intestinal mucosa was provided by Diosynth, Inc. (Chicago, IL). Carrier-free $Na_2{}^{35}SO_4$ was obtained from New England Nuclear (Boston, MA). Nutridoma SP was purchased from Boehringer (Mannheim, Germany). All chemicals were of analytical grade or better. Purified HSPGs with high and low affinity for AT were obtained from RFP culture media and purified by ion exchange, gel filtration, and AT-affinity chromatography. Their protein content was determined using the bicinchoninic acid assay (BCA, Pierce, Rockford, IL) [Kojima et al., 1992a].

Iodination of Antithrombin

AT was labeled with ^{125}I-NaI (15 mCi/μg, Amersham Corp., Arlington Heights, IL), using the chloramine T method [Greenwood et al., 1963]. To protect the heparin-binding site of the protease inhibitor during the labeling procedure, AT was iodinated in the presence of a 10-fold molar excess of affinity-fractionated heparin octasaccharide [Atha et al., 1987]. Heparin octasaccharide was subsequently separated from ^{125}I-AT by gel filtration on a Sephadex G-75 column equilibrated in 50 mM sodium phosphate, pH 7.2, containing 1 M NaCl. Radiolabeled protease inhibitor was then desalted by gel filtration on a Sephadex G-25 column equilibrated in 50 mM sodium phosphate, pH 7.2, containing 0.15 M NaCl and 200 μg/ml bovine serum albumin (BSA). The ^{125}I-AT had a specific activity of 5×10^4 cpm/ng, and was able to bind with avidity to high molecular weight heparin (data not shown).

Cells

The cloned rat fat pad microvascular endothelial (RFP) cells were a generous gift from Dr R. Hoover (Vanderbilt Medical School, Nashville, TN) and have been previously described [Marcum and Rosenberg, 1985]. The cells were grown in M199 medium supplemented with 10% fetal calf serum (GIBCO), 100 units penicillin per ml, and 100 μg of streptomycin per ml. The LTA cell line is derived from mouse L-cells and has been previously characterized [de Agostini et al., 1990b]. The cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum, 100 units penicillin per ml, and 100 μg of streptomycin per ml. For metabolic labeling with $Na_2{}^{35}SO_4$, LTA cells were kept in M199 instead of DMEM. It was verified by ligand binding assay that LTA cells release the same amounts of aHSPGs in DMEM and M199 based conditioned media (data not shown). All cells were maintained in a 95% air/5% CO_2 atmosphere at 37°C.

Conditioned Media Preparation

The cells were seeded at 6×10^4 cells/cm^2 and reached confluence after 3–5 d. Confluent cell monolayers were washed with phosphate-buffered saline (PBS) and incubated 24 h (unless otherwise stated) in serum-free culture medium containing antibiotics. The conditioned media were collected, filtered through 0.22 μm filters or spun at $8,000 \times g$ for 10 min to remove floating cells and debris, and then frozen at −20°C until used. Before testing for aHSPGs, conditioned media were buffered by addition of 1/10 volume of DMEM containing 0.5 M sodium phosphate, pH 7.2. The total amount of protein secreted by cell monolayers (about 10^6 cells) into conditioned media was about 100 μg/ml/24 hr (Coomassie dye binding assay, Biorad).

After collection of conditioned media, the cells were suspended by trypsin treatment and counted on a Coulter counter (Coulter Electronics, Hialeah, FL). The amounts of aHSPGs present within the conditioned media obtained from 10^6 cells were utilized to characterize RFP or LTA cell production of this component. For digestion with *Flavobacterium* heparitinase, conditioned media were dialyzed against distilled water and concentrated 10-fold on a speed-vac evaporator, prior to the enzymatic digestion. The recovery of aHSPGs after concentration was 72% and 56% for RFP and LTA cells, respectively (data not shown).

^{125}I-AT Cell Binding Assay

^{125}I-AT cell binding assay was performed on cells grown in 96 well microtiter plates for 7 d or more. Cell monolayers were preincubated for 1 h at 37°C with media containing 1% Nutridoma SP rather than serum, to eliminate serum-borne AT. The cells were then incubated with ^{125}I-AT diluted in PBS containing 1% Nutridoma SP and 50 μg/ml BSA, with or without a 100-fold excess of unlabeled AT, for 1 h at 4°C.

The unbound ligand was eliminated by washing five times with PBS containing 100 μg/ml BSA, and the cells were removed from the wells using cotton swabs. The swabs were then counted in a gamma-counter (Packard, Downers Grove, IL). Cell numbers were estimated by trypsinizing control wells, and then counting cells on a Coulter counter. All determinations were preformed in triplicate. The values of bound ^{125}I-AT were normalized for 10^6 cells, and nonspecific binding was subtracted. Nonspecific binding was assessed by residual complexed counts in the presence of a 100-fold excess of unlabeled AT, and accounted for 25–30% of the total bound counts for RFP cells and for less than 10% for LTA cells.

^{125}I-AT Ligand Binding Assay

The soluble aHSPGs were adsorbed on 0.22 μm nitrocellulose paper (Millipore) by filtration using a dot-blot apparatus. The blots were subsequently dried for 1 hr at 60°C and the protein binding sites were saturated by a 30 min incubation at 20°C in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 5% nonfat dry milk. The same buffer was used for all subsequent incubations. Following saturation, the blot was incubated for 2 h at 20°C with ^{125}I-AT (1×10^6 cpm/ml, ~ 1 nM) with mild agitation. At the end of the incubation, excess unbound ^{125}I-AT was removed by washing, and the blot was air dried. The blot was then exposed for autoradiography using Kodak X-OMAT AR films (Eastman Kodak, Rochester, NY) with Kodak X-Omatic intensifying screens at −80°C for 18 to 24 h. Bound ^{125}I-AT was quantitated by cutting dots and counting radioactivity in a gamma-counter. All measurements were performed in triplicate. Results were expressed as cpm/well or as cpm/10^6 cells/24 h.

The protein binding capacity of nitrocellulose was estimated to avoid decreased signals due to saturation of the protein binding sites of the membrane. This characteristic of nitrocellulose was defined by comparing ^{125}I-AT binding to soluble aHSPGs from conditioned media containing increasing amounts of either BSA or fetal calf sera. The addition of 50 μg BSA/well did not reduce bound counts, whereas the addition of 100 μg BSA/well produced a 5% decrease in the signal. Fetal calf sera proteins interfered at lower concentrations; 20 μg/well did not reduce bound counts, but 35 μg/well decreased the signal to 90% of the control. Therefore it is possible to use conditioned media containing BSA provided that no more than 100 μg are loaded per well.

Interassay Quantitative Comparison of Conditioned Media aHSPGs

To allow quantitative comparisons between different experiments, pooled LTA conditioned media (LTA pool) was used as an internal standard for quantitation of aHSPGs. ^{125}I-AT ligand binding assays were performed in parallel for purified aHSPGs and LTA pool aliquots, and five independent experiments yielded a constant ratio of 23.1 ± 1.9 between LTA pool (cpm/10^6 cells/24 h) and purified aHSPGs (cpm/ng). LTA pool was routinely incubated in all ^{125}I-AT ligand binding assays, and the signals obtained in individual experiments were normalized using the ratio between the LTA pool value obtained and an average LTA pool value. The average LTA pool value of 32,000 ± 5,000 cpm/10^6 cells/24 h (mean ± SD) was calculated from eight independent experiments ranging from 4 to 22 d after labeling of ^{125}I-AT. The use of LTA pool as an internal standard allows correction for interassay variations due to alterations of the specific activities or binding avidities of ^{125}I-AT.

Quantitation of Cell Surface–Associated aHSPGs

To estimate the relative amounts of cell surface–associated aHSPGs and soluble aHSPGs, we carried out cell binding assays on cell monolayers grown in 96 well titer plates in parallel with ligand binding assays of media collected from the same wells. The cell-bound cpms were normalized using the ratio between LTA pool individual value and LTA pool average value obtained in the matching ligand binding assay as outlined above. The matching ligand and cell binding assays were incubated the same day using identical concentrations of ^{125}I-AT.

Metabolic Labeling of Proteoglycans With $Na_2{}^{35}SO_4$

Confluent monolayers (6 d in culture) were labeled for 24 h with $Na_2{}^{35}SO_4$ (50 μCi/ml) in a mixture of 90% sulfate-free M199 and 10% complete M199 supplemented with 100 μg/ml BSA in the absence of fetal calf sera and antibiotics. Conditioned media were collected and immediately supplemented with 1 mM phenylmethane sulfonylfluoride (PMSF), then centrifuged for 15 min at 8,000 × *g* to remove floating cells. The supernatants were divided in two equal parts, one aliquot examined by gel chromatography

while the other aliquot was filtered through nitrocellulose prior to gel filtration.

Filtration of RFP and LTA Cell Conditioned Media Through Nitrocellulose

The ^{35}S-labeled conditioned media were buffered by addition of 1/10 volume of DMEM containing 0.5 M sodium phosphate, pH 7.2, and subsequently filtered through nitrocellulose using a Millipore filtre support (0.25 ml conditioned medium/cm^2). The effluent was collected and the membranes were washed with the same volume of 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl. The combined filtrates were subsequently concentrated by rotary evaporation and used for gel filtration.

Gel Filtration of ^{35}S-labeled Conditioned Media

The radiolabeled media were concentrated five to 10 times on a speed-vac evaporator and desalted on a Sephadex G-25 column equilibrated in 15 mM sodium phosphate buffer, pH 7.2, containing 50 mM NaCl and 0.3 mM PMSF to eliminate free $Na_2{}^{35}SO_4$. The radiolabeled components eluting in the void volume of the column were recovered and concentrated threefold by rotary evaporation. The samples, with or without prior incubation with chondroitinase ABC (0.1 U/ml, 6 h, 37°C) or with chondroitinase ABC and heparitinase (0.1 U/ml and 0.5 U/ml, respectively, 6 h, 37°C), were loaded on Sephadex G-50 columns in 50 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 1 mM PMSF. The column effluents were monitored for ^{35}S, and degradation of radiolabeled samples was ascertained by comparing the number of counts present in the excluded volume (intact proteoglycans or glycosaminoglycans) with those located in the included volume (oligosaccharide fragments). ^{35}S-labeled components from chondroitinase ABC–treated media were recovered in the excluded volume of the Sephadex G-50 column, concentrated, and loaded on a Sepharose Cl4B column (diameter 1 cm, length 60 cm) equilibrated in Tris-HCl 10 mM buffer, pH 7.4, containing 5 M guanidine HCl and 1 mM PMSF. The effluents were collected in 1 ml fractions and ^{35}S was quantified. The recoveries in ^{35}S counts excluded from the Sephadex G-25 column and then eluted from Sepharose Cl4B were 56 ± 4% (n = 4) and 78 ± 27 (n = 3), and recoveries of ^{35}S counts eluted from Sepharose CL4B were 86 ± 15% (n = 13) and 88 ± 12% (n = 9) for RFP and LTA cells, respectively.

RESULTS

Cultured RFP and LTA Cells Release Soluble aHSPGs Into the Culture Media

The endothelial cell line (RFP) and the fibroblast derived cell line (LTA) were tested for release of soluble aHSPGs into the culture media using the ligand binding assay. Both cell lines were observed to release significant amounts of soluble aHSPGs. The presence of aHSPGs in the conditioned media from RFP and LTA cells was demonstrated by incubating constant amounts of immobilized aHSPGs from 24 h conditioned media (200 μl/well and 50 μl/well for RFP and LTA cells, respectively) with various concentrations of ^{125}I-AT and quantitating bound protease inhibitor (Fig. 1). The binding of AT to the membrane-adsorbed aHSPGs was saturable, and analysis of the data revealed a Kd of 18 and 8 nM for RFP and LTA cells, respectively [Letourneur and Jozefowicz, 1992]. These values are in excellent agreement with those obtained for cell surface–associated aHSPGs [de Agostini et al., 1990a,b].

Specificity of ^{125}I-AT Binding to Immobilized aHSPGs

The specificity of ^{125}I-AT binding to immobilized aHSPGs was examined using LTA rather than RFP conditioned media, because of its stronger signal in ligand binding assays and the similar structure of AT binding sites on aHSPGs from the two cell lines [de Agostini et al., 1990b]. Several lines of evidence demonstrate that AT binding to nitrocellulose-immobilized aHSPGs is specific. First, unlabeled AT competes with radiolabeled AT for binding to immobilized aHSPGs. Thus only 3% of counts bound when ^{125}I-AT was incubated alone were retained in the presence of 1 μM unlabeled AT. Second, AT binding sites are destroyed by *Flavobacterium* heparitinase. After pretreatment for 3 h at 37°C with 0.5 U/ml of heparitinase, conditioned media retained only 1% AT binding capacity of the untreated control. In contrast, preincubation of conditioned media with chondroitinase ABC did not alter AT binding sites present in conditioned media. Third, heparin, but not dextran sulfate, competes with *immobilized* aHSPGs for AT binding. AT binding to immobilized aHSPGs was reduced to 2% of its control value when 10 μg/ml heparin was added to the ^{125}I-AT incubation mixture, whereas 98% of the control binding was observed when the same

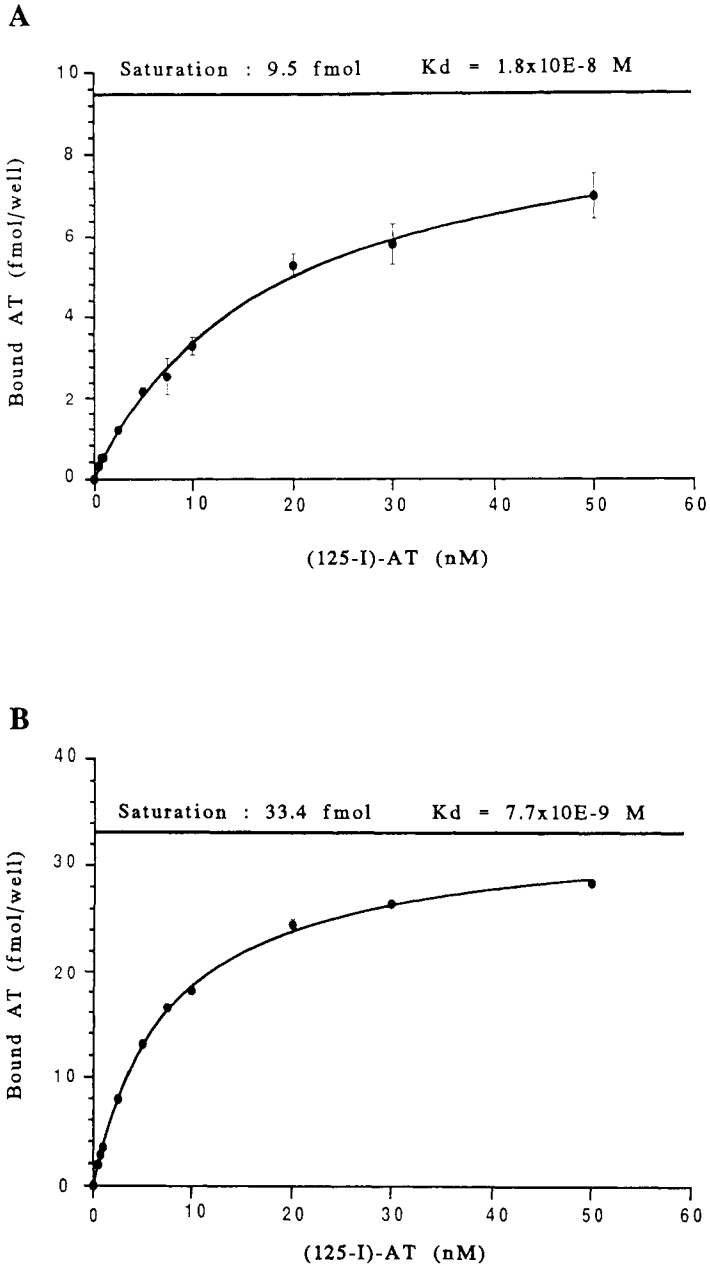


Fig. 1. Binding of ^{125}I-AT to soluble aHSPGs. **A:** RFP cells; **B:** LTA cells. Conditioned media from RFP cells (200 µl/well) and LTA cells (50 µl/well) were immobilized on nitrocellulose and incubated with increasing amounts of ^{125}I-AT (4.4×10^8 cpm/nmole) as outlined in the text. Mean ± SD (n = 3).

concentrations of dextran sulfate were added to the ^{125}I-AT incubation mixture.

Furthermore, the presence of large amounts of heparin within the conditioned media does not interfere with specific detection of aHSPGs. Table I shows that addition of 2 mg/ml heparin to conditioned media did not alter the adsorption of aHSPGs to nitrocellulose. During the saturation of the membrane prior to incubation with AT, soluble heparin is washed away and does not interfere with subsequent binding of ^{125}I-AT to membrane-bound aHSPGs. These data suggest that soluble aHSPGs released by cells interact with nitrocellulose through core protein, whereas commercial heparin, which for the most part lacks covalently attached polypeptide,

TABLE I. Ligand Binding Assay: Detection of aHSPGs in the Presence of Heparin*

Sample adsorbed on nitrocellulose	cpm/well	%
Conditioned media	2,177 ± 46	100
Conditioned media + heparin	2,384 ± 6	112
Heparin	147 ± 20	7

*LTA conditioned media (100 μl/well) was adsorbed on nitrocellulose either alone or in the presence of added heparin (2 mg/ml). Heparin alone (100 μl/well) was 2 mg/ml. After saturation of protein binding sites, the blot was incubated with ^{125}I-AT as described in the Methods section.

is essentially unable to bind to the membrane. This surmise was strengthened by showing that the loading of heparin on nitrocellulose did not facilitate binding of ^{125}I-AT (Table I) and that ^{35}S-labeled heparan sulfate glycosaminoglycan chains released by extensive tryptic proteolysis bind poorly to nitrocellulose (data not shown).

The purified RFP HSPGs were fractionated by AT affinity chromatography into an AT binding fraction (aHSPGs) and a fraction depleted of aHSPGs (iHSPGs). Increasing amounts of aHSPGs and iHSPGs were adsorbed on nitrocellulose, and ^{125}I-AT binding was quantitated. It is apparent that ^{125}I-AT bound on iHSPGs represents only 1% of that bound on aHSPGs (Fig. 2). These data are in excellent agreement with previous results which show the presence of small amounts of residual aHSPGs within iHSPGs [Kojima et al., 1992a]. Taken together, these data demonstrate that ^{125}I-AT binding on conditioned media immobilized on nitrocellulose constitute specific binding to aHSPGs.

Characterization of ^{35}S-labeled HSPGs Immobilized on Nitrocellulose

The nature of the ^{35}S-labeled HSPGs in RFP and LTA cell conditioned media was assessed by digestion with chondroitinase ABC and heparitinase followed by separation on Sephadex G-50 as described in Methods (Table II). The amounts of glycosaminoglycans were estimated as the fraction of ^{35}S-labeled material degraded by combined treatment with chondroitinase ABC and heparitinase to low molecular weight oligosaccharides. The amounts of heparan sulfate chains were calculated as the fraction of ^{35}S-labeled material which could be degraded by heparitinase but which was resistant to the action of chondroitinase ABC. Heparan sulfate chains represent about 50% of RFP- and 90% of LTA-labeled glycosaminoglycans. Parallel analysis showed that heparan sulfate chains constitute about 57% and 75%, respectively, of the nitrocellulose-filtered RFP- and LTA-labeled glycosaminoglycans. These data indicate that similar amounts of soluble heparan and chondroitin sulfate proteoglycans are bound by nitrocellulose.

The molecular size distribution of soluble HSPGs from RFP and LTA cells before and after filtration through nitrocellulose was determined by gel chromatography on Sepharose CL4B (Fig. 3). The HSPGs from RFP conditioned media were eluted as two overlapping peaks (K_{av} 0.47 and 0.84) corresponding to proteoglycans of high molecular size and glycosaminoglycan chains of low molecular size (Fig. 3A, ▲). In contrast, soluble HSPGs from LTA cells eluted as a single broad peak with K_{av} ranging from 0.4 to 0.6 (Fig. 3B, ▲). After filtration through nitrocellulose, the conditioned media contain about 53% of RFP (Fig. 3A, △) and 26% of LTA soluble HSPGs (Fig. 3B, △) and display molecular size distributions identical to the corresponding media before adsorption to the membrane. Therefore, HSPGs bound to nitrocellulose are representative of soluble HSPGs from RFP and LTA cells. We note that a fraction of soluble HSPGs from RFP cells exhibits a molecular size similar to that of free glycosaminoglycan chains but is still able to bind to nitrocellulose. These data imply that the free glycosaminoglycan chains possess sufficient peptide to allow adsorption to the membrane.

Kinetics of Release of aHSPGs in Conditioned Media

In order to determine whether the time of exposure of cells to serum-free media altered the release of aHSPGs, we assayed media collected after varying periods of incubation. Figure 4 shows that aHSPGs from both cell types are continuously released in the media over 72 h. The amounts of released aHSPGs were detectable in the media after 6 h and increased linearly with time. Moreover, similar results occurred after three successive collections of 24 h serum-free conditioned media, demonstrating that sustained release of soluble aHSPGs takes place for several days independent of serum factors.

The Time-Dependent Partition of aHSPGs Between Cell Layers and Media

The time-dependent partition of aHSPGs between cell layers and culture media was deter-

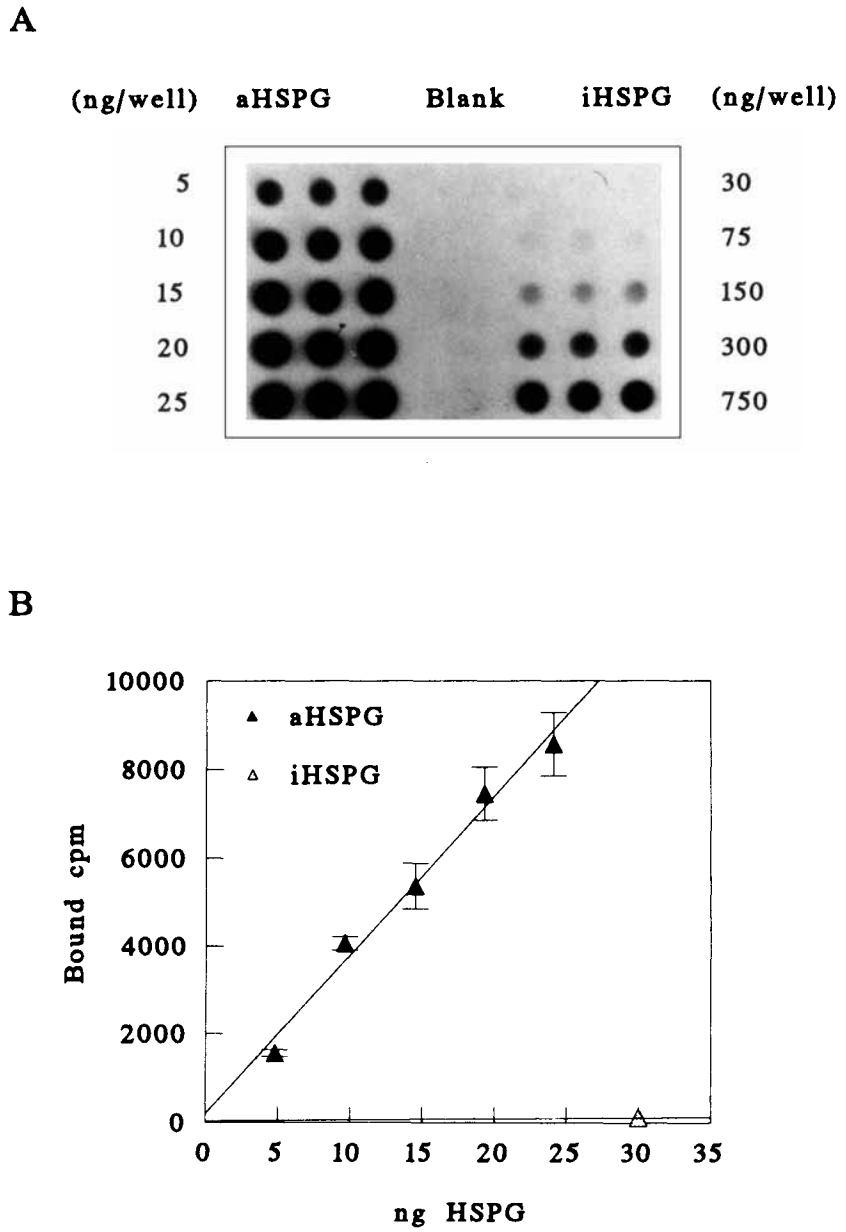


Fig. 2. ^{125}I-AT binding to purified aHSPGs and iHSPGs. Known amounts of aHSPGs and iHSPGs were loaded on nitrocellulose and incubated with ^{125}I-AT. The blot was then exposed for autoradiography for 15 hr. The dots were then excised and radioactivity counted. **A:** Autoradiograph of the blot. **B:** Quantification of bound AT. Mean ± SD (n = 3).

mined by carrying out parallel measurements of cell surface–associated aHSPGs and soluble aHSPGs. The RFP and LTA cells were seeded in parallel cultures at 20,000 cells per well in 96 well plates and grown to confluence. At various times after confluence, the monolayers were incubated for 24 h in serum-free media to generate conditioned media, and subsequently aHSPGs were measured by ^{125}I-AT ligand binding and cell binding assays, respectively (Figs. 5 and 6). The levels of cell surface–associated and soluble aHSPGs of RFP cells increased with time in culture. The magnitude of the increase was greater for soluble aHSPGs than cell surface–associated aHSPGs as shown by the slopes of the linear regression curves for soluble aHSPGs in 24 h conditioned media (Fig. 6: $y = 1.733x + 0.905$) as compared to cell surface–associated aHSPGs (Fig. 5: $y = 0.463x + 7.055$). In contrast, LTA cells generate constant amounts of cell surface–

TABLE II. Sephadex G-50 Elution of RFP and LTA Conditioned Media

	^{35}S cpm (%)					
	RFP			LTA		
Enzyme	Excluded volume PGs and GAGs	Included volume oligosaccharides	Total	Excluded volume PGs and GAGs	Included volume oligosaccharides	Total
—	93	7	100	99	1	100
Chondroitinase ABC	53	47	100	92	8	100
Chondroitinase ABC + heparitinase	11	89	100	19	82	100

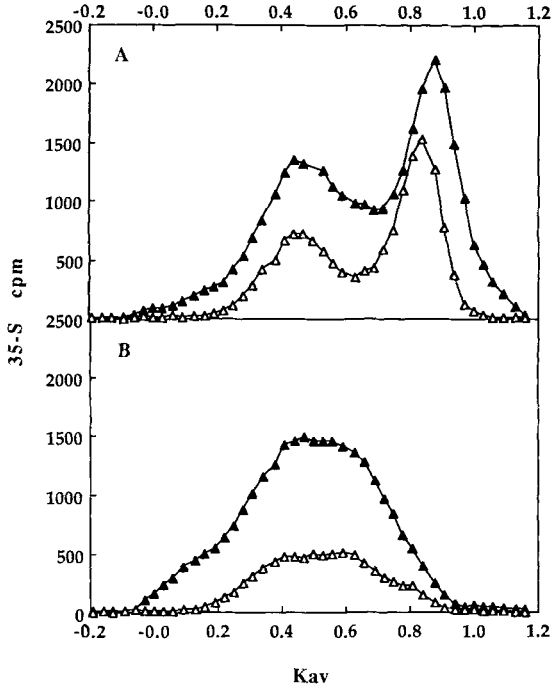


Fig. 3. Gel filtration of ^{35}S-labeled RFP and LTA cell soluble HSPGs on Sepharose CL4B. Standards used for calibrating the column were Blue Dextran (K_{av} = 0.00), Dextran T70 (K_{av} = 0.6), Heparin (K_{av} = 0.75), Dextran sulfate M_r 5,000 (K_{av} = 0.94), and $Na_2{}^{35}SO_4$ (K_{av} = 1.00). **A:** (solid triangles) HSPGs from RFP conditioned media, (open triangles) HSPGs from nitrocellulose-filtered RFP conditioned media; **B:** (solid triangles) HSPGs from LTA conditioned media, (open triangles) HSPGs from nitrocellulose-filtered LTA conditioned media. Values displayed are from a representative analysis and similar values were obtained from two independent sets of conditioned media.

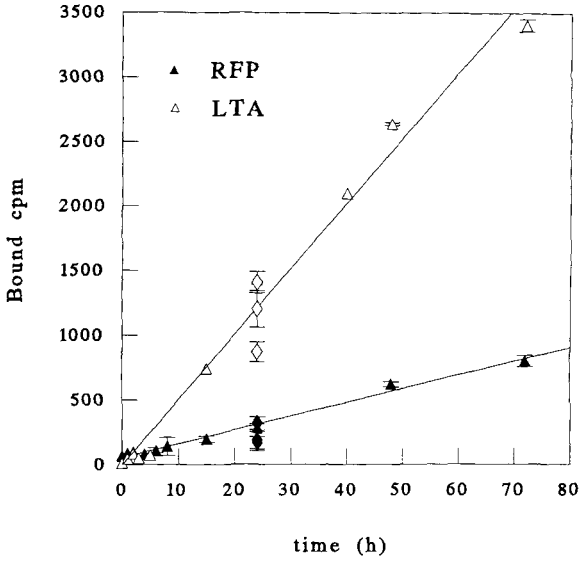
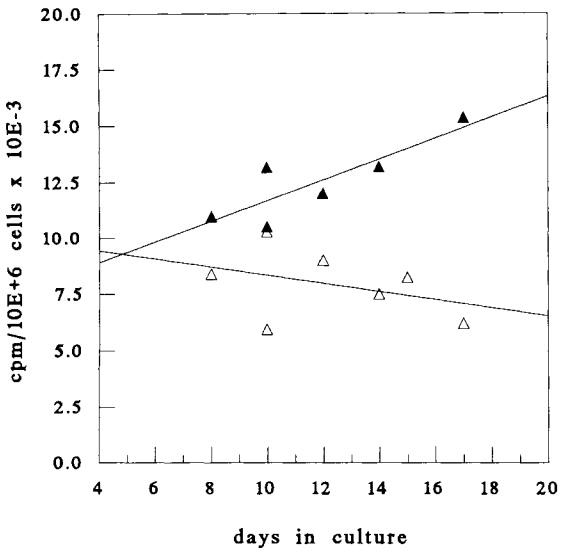


Fig. 4. Kinetics of release of aHSPGs in conditioned media. Ligand binding assay of media conditioned for various times on RFP and LTA cells. Serum-free media were conditioned on cell monolayers either for different times (solid triangles) or successively for three 24 hr periods of time (solid diamonds) prior to testing.

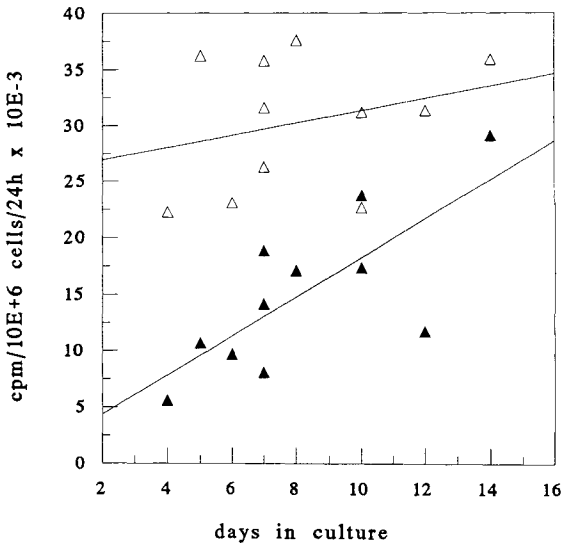
associated aHSPGs over time, and release constant amounts of soluble aHSPGs. We note that the amounts of cell surface–associated aHSPGs on RFP cells are greater than those on LTA cells, whereas LTA cells secrete greater levels of soluble aHSPGs than RFP cells (Table III).

The relative daily accumulation of cell surface–associated aHSPGs as compared to the total daily accumulation of aHSPGs for postconfluent RFP cells can be determined from Table II. We estimate that postconfluent RFP cells maintain about 1–2% of the total daily accumulation of aHSPGs as cell surface–associated aHSPGs. For comparison, postconfluent LTA cells do not accumulate measurable amounts of aHSPGs in their cell layers. Finally, we can also ascertain the levels of cell surface–associated aHSPGs at a given point in time as compared to the total 24 h accumulation of aHSPGs. After 8 d in culture, the levels of cell surface–associated aHSPGs represent 63% and 22% of the daily accumulation of aHSPGs for RFP and LTA cells, respectively. These data suggest that confluent RFP and LTA cells generate sufficient aHSPGs to completely restore cell surface–associated aHSPGs in less than 24 h. The above prediction has previously been confirmed for RFP cells



▲ RFP y = 7.055 + 0.463x Corr = 0.850
△ LTA y = 10.158 - 0.181x Corr = 0.379

Fig. 5. Time-dependent accumulation of cell surface–associated aHSPGs by RFP and LTA cells. Cell monolayers were kept in culture for various times after which cell surface–associated aHSPGs were quantified by ^{125}I-AT cell binding assay. Each point represents a separate experiment.



▲ RFP y = 0.905 + 1.733x Corr = 0.745
△ LTA y = 25.870 + 0.548x Corr = 0.283

Fig. 6. Time-dependent accumulation of soluble aHSPGs by RFP and LTA cells. Soluble aHSPGs were quantified by ^{125}I-AT ligand binding assay in 24 hr conditioned media collected after various times in culture. Each point represents a separate experiment.

after extensive trypsinization [de Agostini et al., 1990a], and similar behavior has been observed for LTA cells (data not shown).

DISCUSSION

The availability of assays to measure cell surface–associated aHSPGs as well as soluble aHSPGs allowed us to investigate the distribution of these components in different cell types. Our results demonstrate that RFP and LTA cell lines retain significant quantities of cell surface–associated aHSPGs but also release significant amounts of soluble aHSPGs into culture media. The postconfluent RFP cells exhibit a time-dependent accumulation of cell surface–associated aHSPGs as well as a time–dependent augmentation in release of soluble aHSPGs. The postconfluent LTA cells exhibit a constant level of cell-associated aHSPGs as well as a constant extent of release of soluble aHSPGs. After trypsin treatment RFP cells have been shown to restore cell-associated aHSPGs within 12 h [de Agostini et al., 1990a] and LTA cells displayed the same behavior (data not shown). The aHSPGs of RFP cells exhibit transmembrane domains and cytoplasmic C-terminal extensions in addition to large extracellular glycosylated domains [Kojima et al., 1992b]. Therefore, we suspect that the initial rapid accumulation of cell surface–associated aHSPGs detected by the ^{125}I-AT cell binding assay probably represents membrane inserted proteoglycans. These membrane intercalated aHSPGs may subsequently be released to either diffuse freely and accumulate in the media or bind to extracellular matrix and be replaced on the cell membrane by newly synthesized aHSPGs.

The aHSPGs of RFP cells have been identified as syndecan and the 30 kD ryudocan [Kojima et al., 1992b]. Human aortic and umbilical vein endothelial cells synthesize five different membrane-bound HSPGs with core proteins exhibiting molecular sizes ranging from 130 kD to 35 kD as well as a basement membrane HSPG, all of which bind AT [Mertens et al., 1992]. Four of these endothelial HSPGs, identified as perlecan, fibroglycan, glypican, and syndecan, are present both on endothelial cells and fibroblasts [Heremans et al., 1988; Mertens et al., 1992; Lories et al., 1992]. Moreover, Mertens et al. have identified glypican and perlecan in human umbilical vein endothelial cell culture medium. The attachment of AT binding heparan sulfate chains to different core proteins suggests that the ob-

TABLE III. aHSPGs Partition at Different Times in Culture

Days in culture	Cell layers (cpm/10^6 cells)		Conditioned media (cpm/10^6 cells/24 hr)	
	RFP	LTA	RFP	LTA
8	10,960 ± 594	8,385 ± 528	17,091 ± 1,124	37,601 ± 2,041
14	13,191 ± 2,211	7,498 ± 1,093	29,121 ± 3,568	35,902 ± 4,521

Cells were grown in 96 well titer plates. Soluble aHSPGs was detected in conditioned media incubated 24 hr before testing for cell surface aHSPGs.

served difference in the behavior of aHSPGs in postconfluent RFP and LTA cells could be due to expression of different sets of core proteins or altered processing of the same set of core proteins. It seems unlikely that the observed variations in the behavior of aHSPGs in the rat RFP and mouse LTA cells are due to species differences. The same mammalian HSPGs found in different species exhibit extensive sequence homologies as evidenced by perlecan [Kallunki and Tryggvason, 1992] and syndecan [Cizmeci-Smith et al., 1992]. In contrast, HSPGs are known to be expressed in cell type–specific patterns [Lories et al., 1992] and to vary according to developmental stage [Brauker et al., 1991], hormonal stimulation [Morris et al., 1988], and tissue-specific induction [Boutin et al., 1991].

We have demonstrated that extremely small amounts of soluble aHSPGs immobilized on nitrocellulose can be quantitated by our ligand binding assay. Jalkanen and collaborators have described an immunodot assay for syndecan immobilized on DEAE paper [Jalkanen et al., 1985]. These authors measured with a monoclonal antibody syndecan core protein bound via its glycosaminoglycan chains to DEAE-cellulose paper and reported that syndecan does not adsorb to nitrocellulose. The limit of detection of their assay is about 0.5 μg of protein/dot. In our ligand binding assay, we quantitate the AT binding sites present on glycosaminoglycan chains of aHSPGs bound to nitrocellulose via core proteins. The limit of detection of purified aHSPGs bound to nitrocellulose is about 0.5 ng protein/well and AT binding to aHSPGs increases linearly with the amount of aHSPGs applied up to 30 ng protein/well. At higher levels of aHSPGs, the binding of AT reaches a plateau indicating saturation of the nitrocellulose. The nitrocellulose binding capacity was found to differ according to which proteins are adsorbed as demonstrated by the fact that 20 μg of serum proteins and 100 μg of BSA did not interfere with adsorption of aHSPGs. The lower binding capacity of nitrocellulose for proteoglycans is probably related to the large size of these molecules and to the hydrophilic nature of their glycosaminoglycan chains. Nevertheless, the exquisite specificity of AT binding to aHSPGs permits us to quantify nanogram amounts of soluble aHSPGs directly from cell conditioned media.

It has also been reported that mouse mammary epithelial cell proteoglycans bind quantitatively to cationized nylon, while only 50% are retained on DEAE paper and less than 1% is retained on nitrocellulose [Rapraeger and Yeaman, 1989]. These observations raise the issue that particular subsets of HSPGs might bind to nitrocellulose. We have carried out gel filtration analyses of radiolabeled HSPGs from RFP and LTA cell conditioned media and demonstrate that 47% and 74%, respectively, of total HSPGs are retained on nitrocellulose. The radiolabeled HSPGs bound to nitrocellulose exhibit a molecular size distribution identical to that present in conditioned media. Thus, a significant fraction of the small amounts of soluble HSPGs in culture media binds to nitrocellulose and appears to be representative of HSPGs present in culture media. We have observed more quantitative binding of ^{35}S-labeled species on cationized nylon as compared to nitrocellulose, but have found it impossible to perform AT binding assays on this membrane because of a poor signal-to-noise ratio (data not shown). Therefore, the sensitivity of our ^{125}I-AT ligand binding assay permits us to employ nitrocellulose to bind HSPGs despite the relatively low capacity of this material to adsorb proteoglycans.

The demonstration that cultured cell lines not only retain aHSPGs on their cell surfaces and within their extracellular surroundings but also release considerable amounts of these components into their surrounding environment suggests a possible distal function for these macromolecules. Two possible scenarios may be constructed in which release of aHSPGs might serve an important physiologic function. On the

one hand, endothelial cells could liberate aHSPGs into interstitial fluid and then into the blood stream, which would allow these components to regulate plasma AT and hence control the action of the coagulation mechanism. It is of interest to note that rare instances have been described of circulating heparin-like anticoagulants associated with various pathologic situations such as multiple myeloma and bladder carcinoma [Khoory et al., 1980; Palmer et al., 1984; Tefferi et al., 1989]. Horne and colleagues have also reported the presence of circulating anticoagulant glycosaminoglycans in patients with liver damage subsequent to suramin treatment of tumors [Horne et al., 1988]. Therefore, it seems possible that aHSPGs may be normally present in the blood at levels which cannot be detected with current methodology.

Alternatively, vascular cells may release aHSPGs into the interstitial cell fluid with subsequent binding to basement membrane just beneath the endothelial cell surface. This would allow the accumulation of substantial amounts of aHSPGs on the abluminal surface of the endothelium as previously documented by us with EM level radioautography of intact blood vessels [de Agostini et al., 1990a]. Plasma AT should have relatively free access to this locale, as suggested by numerous studies which document the extraordinary permeability of the endothelial cell layer [Simionescu, 1983]. The presence of this subendothelial concentration of aHSPGs would also explain kinetic radiotracer studies that suggest that a significant amount of labeled protease inhibitor is located in a unique extravascular compartment [Carlson et al., 1985]. The interaction of coagulation enzymes with AT bound to subendothelial aHSPGs could constitute the heparin-like, macromolecule-dependent acceleration of protease inhibitor action observed in animal models [Marcum et al., 1984]. This would imply that inhibition of coagulation enzymes is quite active within subendothelial regions, and would expand our present notions of the critical biologic surfaces which are in contact with the hemostatic mechanism. It seems quite possible that both scenarios are functional under in vivo conditions with the release and partition of aHSPGs playing a critical role in maintaining blood fluidity. The mechanism that is responsible for the release and partition of aHSPGs is poorly understood at the present time and requires further characterization.

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