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Novel Purification and Detailed Characterization of Perlecan Isolated from the Engelbreth-Holm-Swarm Tumor for Use in an Animal Model of Fibrillar Aβ Amyloid Persistence in Brain[1]

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Co-infusion of the specific heparan sulfate proteoglycan (HSPG), perlecan, and beta-amyloid protein (Aβ) into rodent hippocampus leads to a consistent animal model to study the effects of fibrillar Aβ amyloid in brain [Snow, A.D. *et al.* (1994) *Neuron* 12, 219-234]. In the present study, we describe our rapid novel method of perlecan isolation. The isolation method does not require cesium chloride centrifugation and exploits a newly discovered aggregating property of a ~220 kDa PG observed during gel filtration chromatography, which allowed it to be affectively separated from non-aggregating perlecan. Fifty or 100 g of EHS tumor were routinely extracted using 4 M guanidine-HCl, followed by anion-exchange and gel filtration chromatography. SDS-PAGE (before and after digestion with heparitinase/heparinase or nitrous acid) followed by staining with silver demonstrated no other contaminating proteins in the perlecan preparations. Western blots using a specific perlecan core protein antibody (HK-102) following heparitinase digestion showed a characteristic doublet at 400 and 360 kDa indicative of intact perlecan core protein. Absence of contamination by other basement membrane components produced by the EHS tumor was confirmed by absence of immunoreactive bands on Western blots using antibodies against laminin, fibronectin, or type IV collagen. One week continuous co-infusion of perlecan obtained from this methodology, with Aβ (1-40) into rodent hippocampus, led to deposition of fibrillar Aβ amyloid in 100% (10 of 10) of animals. The detailed protocol for isolation and characterization of perlecan from EHS tumor ensures perlecan of the highest quality, and maximizes the potential effects of Aβ amyloid deposition/persistence in brain using the animal model. High quality perlecan obtained from this novel isolation method will also allow future studies utilizing *in vitro* assays to determine the potential interactions of this specific HSPG with other macromolecules.

Key words: Alzheimer's disease, EHS tumor, heparan sulfate, perlecan, proteoglycans.

Proteoglycans (PGs) consist of a protein core to which are attached at least one covalently linked glycosaminoglycan (GAG) chain. Different classes of PGs exist in all tissues and organs and are synthesized by most, if not all, cells. PGs are generally classified according to the type of GAG chain present and different major classes exist including heparan sulfate proteoglycans (HSPGs), chondroitin sulfate proteoglycans, dermatan sulfate proteoglycans, and keratan sulfate proteoglycans (*1*). In addition, PGs may exist as hybrid macromolecules, containing two different types of GAGs present on the same core protein (*2, 3*).

One specific HSPG, known as perlecan, is present on all basement membranes (*4-6*) and was previously cloned from both human (*7, 8*) and mouse (*9*). Perlecan is produced by different cell types including endothelial cells (*10-12*), smooth muscle cells (*13*), fibroblasts (*6, 14*), epithelial cells (*15-17*), and synovial cells (*18*). Perlecan is also synthesized by bone marrow derived cells (*19*) and is present in cancerous tissue including metastatic melanomas (*20*), human breast tumors (*21*), and liver tumors (*22*). Both F9 embryonal carcinoma cells (which form parietal endoderm) and P19 embryonal carcinoma cells (which form cholinergic neurons) also demonstrate marked increased perlecan expression and synthesis upon differentiation (*23, 24*).

Perlecan is postulated to play a primary role in the pathogenesis of Alzheimer's disease (AD) amyloidosis, as well as in other types of central nervous system and systemic amyloidoses (reviewed in Ref. *25*). Recent studies indicate (*26*) that both the core protein and GAG chains of perlecan participate in binding to the Alzheimer's disease beta-amyloid protein (Aβ). Once bound, perlecan is believed to alter the secondary structure (*27*) and enhance the aggregation and fibrillogenesis properties of Aβ (*28*). In addition, perlecan protects Aβ from protease degradation *in vitro* (*29*) and *in vivo* (*30, 31*). Co-infusion of perlecan

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Abbreviations: Aβ, beta-amyloid protein; AD, Alzheimer's disease; CHAPS, 3-[3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; DD, double distilled water; EHS, Engelbreth-Holm-Swarm; GAGs, glycosaminoglycans; HSPG, heparan sulfate proteoglycan; NEN, *N*-ethylmaleimide; PGs, proteoglycans; PMSF, phenylmethylsulfonyl fluoride; TTBS, Tris-buffered saline containing 0.05% Tween-20.

and Aβ (1-40) into rat hippocampus led to consistent fibrillar Aβ amyloid deposits, which were otherwise variable in animals infused with only Aβ (*30*). This co-infusion of Aβ + perlecan has provided us with a consistent animal model in which to study the effects of persistent fibrillar Aβ amyloid deposits in brain and to screen for potential therapeutic agents targeting Aβ amyloid persistence in brain. However, for use in this animal model, the purity of the perlecan preparation being infused appears to be important for the extent and consistency of fibrillar Aβ amyloid deposition and persistence in brain (Snow, Cummings, and Castillo, unpublished data). Contaminating basement membrane proteins such as laminin, fibronectin, and type IV collagen, which normally associate with perlecan (*32*), can cause variability in the animal model. This may be attributed to the masking of perlecan binding sites involved in binding to Aβ, and/or masking of Aβ binding sites (*i.e.* residues 12-17) (*26*) involved in binding to perlecan.

The most commonly utilized source for isolation of perlecan is the Engelbreth-Holm-Swarm (EHS) tumor which is routinely grown in the hind legs of mice (*2, 33-35*). Previous methods of perlecan isolation from the EHS tumor have involved a series of extractions and cesium chloride centrifugation (*2, 33-36*). Cesium chloride density gradient centrifugation was previously included to separate the two major PGs produced by the EHS tumor, namely perlecan (referred to as the low density HSPG), and a smaller HSPG of 200-400 kDa (referred to as the high density HSPG) (*2, 33-36*), which has not been fully characterized. Some of the major problems in using these previous methods of perlecan isolation include: (a) contamination by other proteins and/or basement membrane components (*i.e.* laminin, fibronectin and type IV collagen) produced by the EHS tumor, (b) contamination due to the presence of free GAG chains, and (c) degradation of the perlecan core protein.

In the present investigation, we describe a relatively rapid and novel method of perlecan purification at preparative amounts. This method exploits a newly discovered aggregating property of the ~220 kDa PG observed during gel filtration chromatography, which was not exhibited by perlecan. In addition, this purification protocol employs a higher pH than previously recommended (*2, 33, 34*), which is believed to aid in the prevention of protein-GAG interactions (*37*), therefore decreasing the involvement of possible contaminants which may bind to perlecan during fractionation. The perlecan preparations obtained from this novel methodology were of consistent high quality as demonstrated by an intact core protein, and absence of any contaminating proteins and/or free GAG chains. Infusion of this perlecan product into rodent brain, in the presence of Aβ, consistently led to fibrillar Aβ amyloid deposits in brain in 100% of animals, which was only observed in 60% of animals following infusion of Aβ alone. The use of high quality perlecan for (a) consistent *in vivo* Aβ amyloid deposition, accumulation and persistence in rodent brain, and (b) the development of new *in vitro* assays should accelerate our understanding of the many roles this specific HSPG may be involved in.

MATERIALS AND METHODS

Materials—C57BL mice were purchased from B&K Universal (Kent, WA). Nembutal was from Abbott Laboratories (North Chicago, IL). Alcian Blue, Coomassie Blue, EHS laminin, bovine plasma fibronectin, EHS type IV collagen, affinity purified rabbit polyclonal anti-laminin (L9393), heparinases I, II, and III, BSA, normal goat serum, guanidine-HCl, CHAPS, Tris-HCl, *N*-ethylmaleimide (NEM), 6-aminohexanoic acid, benzamidine-HCl, phenylmethylsulfonyl fluoride (PMSF), silver nitrate, sodium bicarbonate, sodium nitrite, and sodium citrate were all purchased from Sigma Chemical (St. Louis, MO). Methanol, potassium acetate, glutaraldehyde, sodium hydroxide, ammonium hydroxide, sodium azide, formaldehyde, acetic acid, sodium chloride, (ethylenediamine) tetraacetic acid (EDTA), and urea were all from J.T. Baker (Phillipsburg, NJ). Absolute ethanol was from McCormick (Pekin, IL). Sephacryl S-400 and S-1000, DEAE-Sephacel, and all columns used for analysis were purchased from Pharmacia (Uppsala, Sweden). Triton X-100 was from Boehringer Mannheim (Indianapolis, MN).The conductivity meter with Model number 2052 was from VWR Scientific (Seattle, WA). The Mini-Protean II electrophoresis system, mini transblot electrophoresis transfer cell, pre-cast polyacrylamide gradient gels (4-15%), electrophoresis running buffer, SDS sample buffer, and prestained molecular weight protein standards were from Bio-Rad (Richmond, CA). Nitrocellulose (0.45 μm) was from Schleicher and Schuell (Keene, NH). Anti-laminin polyclonal antibody (AB756) and anti-fibronectin polyclonal antibody (AB1941) were from Chemicon (Temicula, CA). Biotinylated secondary antibodies (goat anti-rat and goal anti-rabbit) were purchased from Jackson Immuno-Research (West Grove, PN). Avidin alkaline phosphatase conjugate and alkaline phosphatase substrate solution (Vectastain ABC kit) were from Vector Labs (Burlingame, CA). Tween-20 was from Calbiochem (La Jolla, CA). Anti-perlecan core protein monoclonal antibody (HK-102) was a generous gift from Dr. Koji Kimata (Japan).

Purification of Perlecan from the Engelbreth-Holm-Swarm Tumor—Figure 1 shows the general protocol for perlecan purification from the EHS tumor. The EHS tumor was maintained in the right or left hind leg muscle of C57Bl mice following injection of tumor cells as previously described (*38-40*). The tumors were usually maintained in the mice hind legs for 3-4 weeks usually attaining a growth of approximately 3-4 g. In accordance with NIH Animal Care and Use Guidelines, the animals were sacrificed by lethal injection of Nembutal (0.50 ml of 50 mg/ml solution per mouse), before the tumor tissue reached an approximate weight of 4 g. EHS tumor tissue was harvested from the mice as previously described (*40*). All extraction steps (described below) were carried out by agitation using a rotary shaker at 600 rpm (Model M65825, Barnstead/Thermolyne, Dubuque, IA). The tumor tissue (50 g at a time) was routinely minced and extracted with 2.5 tissue volumes of 4 M guanidine-HCl, 0.5% (w/v) CHAPS, 50 mM Tris-HCl (pH 7.5) containing a protease inhibitor cocktail including 10 mM EDTA, 10 mM NEM, 10 mM 6-aminohexanoic acid, 5.0 mM benzamidine-HCl, and 1 mM PMSF for 3 h at 4°C. The supernatants were collected

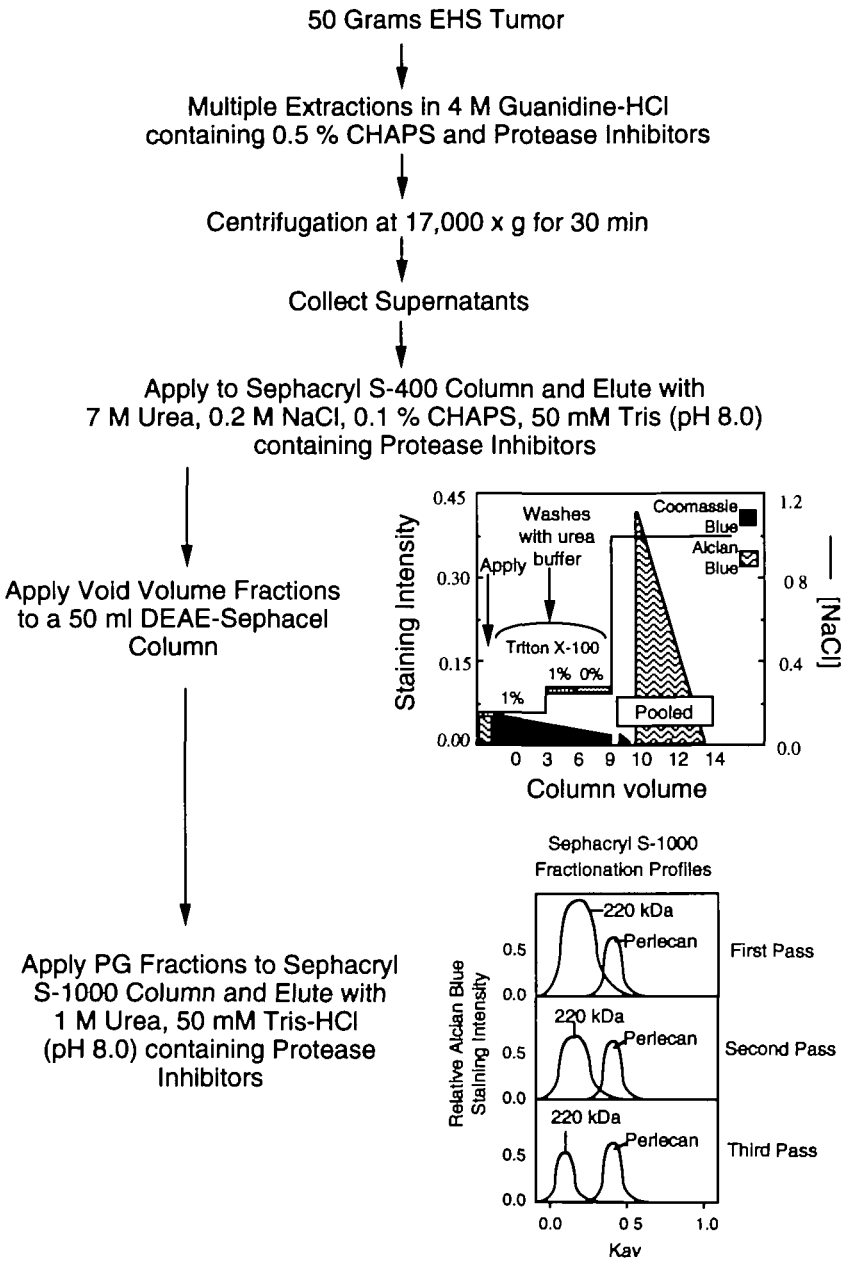

Fig. 1. **General protocol for perlecan purification from the Engelbreth-Holm-Swarm tumor.** This figure demonstrates the general protocol employed for the isolation of perlecan from EHS tumor tissue. In general, tumor tissue (50 g per run) was extracted with 4 M guanidine-HCl containing 0.5% CHAPS, and protease inhibitors, and following centrifugation, the supernatants were pooled and applied to a Sephacryl S-400 column in the presence of 7 M urea buffer. Perlecan and the ~220 kDa PG present in the void volume fractions were pooled and applied to a DEAE-Sephacel column. After initial washes (in 0.25 M NaCl) to remove non-PG proteins, bound PGs were removed by washing in the presence of 3 M NaCl, and then applied to a Sephacryl S-1000 column. Fractions containing perlecan (K_{av}=0.29–0.54) were then pooled, and concentrated on a DEAE-Sephacel column. Following ethanol precipitation, the resulting pellets were dissolved in double distilled water, dialyzed extensively against double distilled water, aliquoted and freeze-dried.

following centrifugation at 17,000×*g* for 30 min. Additional extractions of the remaining pellets was achieved using 1.5 tissue volumes with guanidine-HCl (as described above), first for 2 h, and then overnight. The guanidine extracts were then pooled and applied (80 ml at a time) to Sephacryl S-400 columns (4.8×28 cm). Samples were then eluted using a urea buffer containing 7 M urea, 0.2 M NaCl, 0.1% (w/v) CHAPS, 50 mM Tris-HCl (pH 8.0) containing protease inhibitors as described above. The void volume fractions (believed to contain perlecan) from the Sephacryl S-400 column were then pooled and supplemented with 0.5% Triton X-100 (v/v) and applied to a 50 ml DEAE-Sephacel column packed in a 60 ml plastic syringe equilibrated with urea buffer. Proteins and non-PGs were removed by first washing the column with 3 column volumes of urea buffer containing 1% Triton X-100, followed by 3 column volumes of urea buffer containing 0.25 M NaCl and 1% Triton X-100, and then 3 column volumes of urea buffer containing 0.25 M NaCl without Triton X-100. Bound PGs were then eluted with 5 column volumes of urea buffer containing 1 M NaCl or 3 M NaCl. The 3 M NaCl elutions were used to determine whether tightly bound PGs not eluted with 1 M NaCl still remained bound to the column.

PGs obtained from the DEAE-Sephacel column were first loaded onto a Sephacryl S-500 column (2.6×60 cm) in order to try to separate the 220 kDa PG from perlecan (700–800 kDa). In a preliminary study, we found the Sephacryl S-500 column was not able to properly separate the 220 kDa PG from perlecan (both were found in the void volume fractions), due to the self-aggregating ability of the 220 kDa PG. Even the use of dissociating buffers including, (a) 4 M guanidine-HCl, 0.5% CHAPS, and 50 mM Tris-HCl (pH 7.5), (b) 7 M urea, 0.2 M NaCl, 1% SDS (w/v), and 50

mM Tris-HCl (pH 8.0), and (c) 7 M urea, 0.2 M NaCl, 0.1% CHAPS, and 50 mM Tris-HCl (pH 8.0), were not able to prevent the aggregation of the 220 kDa PG. Separation of the 220 kDa PG from perlecan was finally achieved using a Sephacryl S-1000 column (5×95 cm) under associating conditions (1 M urea buffer containing 50 mM Tris-HCl and protease inhibitors including 1.4 mM EDTA, 1.4 mM NEM, 1.4 mM 6-aminohexanoic acid, 0.7 mM benzamidine, and 0.14 mM PMSF; pH 8.0).

PGs eluted from DEAE-Sephacel were loaded (50 ml per run) onto the Sephacryl S-1000 column (as described above). PGs eluted from the Sephacryl S-1000 column were monitored by SDS-PAGE analysis in order to assess the purity of perlecan and other HSPGs which may be present. For this analysis, 100 μl aliquots of each 60 ml fraction was precipitated with 4 volumes of absolute ethanol by cooling on dry ice for 1 h, and then centrifuged on a microcentrifuge at 12,000×g for 20 min, and run on SDS-PAGE as described below. Pooled fractions (from 5 separate runs) containing perlecan (K_{av}=0.29-0.54) (see "RESULTS") were then concentrated on and eluted from a 15 ml DEAE-Sephacel column with 3 M NaCl, and re-chromatographed onto the Sephacryl S-1000 (as described above). Usually two or three passes through the Sephacryl S-1000 column gave high quality perlecan preparations free from any other contaminating HSPGs (*i.e.* 220 kDa PG) (*33*) or other PGs. The perlecan fractions were pooled and concentrated onto a 10 ml DEAE-Sephacel column and ethanol precipitated (as described above). The resulting pellets were dissolved in 3-5 ml of double distilled water and extensively dialyzed against double distilled water until the conductivity of the end product contained very little to no salt (2-3 μmhos) as measured using a digital conductivity meter. The final perlecan product was then freeze-dried and stored. The final purity of the perlecan preparations were further assessed by Alcian blue staining, Coomassie Blue staining, silver staining, and a series of Western blots (as described below).

SDS-PAGE—SDS-PAGE was performed according to the method of Laemmli (*41*) using a Mini-Protean II electrophoresis system with precast 4-15% polyacrylamide gels. In order to examine perlecan quality on SDS-PAGE following the Sephacryl S-1000 chromatography described above, pelleted fractions were redissolved in 20 μl of 1× reducing SDS sample buffer, heated for 5 min in a boiling water bath, and electrophoresed at 200 V for 45 min along with pre-stained molecular weight protein standards. All gel staining and washing steps were carried out with mild agitation using a rotary shaker.

Alcian Blue, Coomassie Blue, and Silver Staining—Alcian blue staining of SDS-PAGE gels was employed to detect PGs (*42*) using a modified method (Castillo and Snow, unpublished observations). Following electrophoresis, gels were rinsed 3 times (for 20 min each) with 50% methanol, 10% acetic acid (v/v), and then stained for 2 h with 0.1% Alcian blue in 50% methanol and 10% acetic acid. For destaining, the gels were rinsed 6-10 times for 20 min each, with the same solution as described above, but without Alcian blue. Non-PG proteins were visualized by staining for 2 h in the same solution (described above) containing 0.2% (w/v) Coomassie Brilliant Blue and de-staining in a similar manner as in the Alcian Blue staining protocol (described above).

Some gels were also stained with silver to detect any contaminating proteins in our preparations, using the methods previously described (*43*), with slight modifications. Briefly, gels were washed for 25 min in 50% methanol and 10% acetic acid (v/v), followed by three washes (10 min each) in 10% ethanol and 5% acetic acid (v/v), and then two washes (10 min each) with distilled deionized (DD) water. Gels were then fixed for 20-40 min with freshly made 1% (v/v) glutaraldehyde and 0.2 M sodium bicarbonate, and washed twice (10 min each) with DD water. The gels were then stained for 30 min with freshly made 0.8% $AgNO_3$ (w/v), 0.07% NaOH (w/v), 1.3% NH_4OH (v/v), and 15% ethanol (v/v), rinsed three times (10 min each) with DD water, and developed using a solution containing 0.005% sodium citrate (w/v), 0.037% formaldehyde (v/v), and 10% ethanol (v/v). The silver reaction was stopped by adding 5% acetic acid (v/v).

Digestion with Heparitinase/Heparinase and Nitrous Acid—Prior to SDS-PAGE, some of the ethanol precipitated HSPGs were digested by incubation overnight at 41°C with 1.0 unit each of heparinases I, II, and III (heparitinase) in 90 μl of digestion buffer consisting of 100 mM Tris-HCl, 2.5 mM calcium acetate, 5.0 mM 6-aminohexanoic acid, 2.5 mM benzamidine-HCl, 5.0 mM NEM, and 0.50 mM PMSF (pH 7.0). The next day, digested samples were precipitated by adding 3.5 volumes of 95% ethanol, 1.5% potassium acetate (w/v), cooled on dry ice for 1 h and then centrifuged at 12,000×g for 20 min. For Western blotting of perlecan core protein, the pellets were redissolved in 20 μl of non-reducing SDS sample buffer (since reduction eliminates the antigenic sites recognized by anti-HK-102) (*5*).

In addition, prior to SDS-PAGE, some of the ethanol precipitated HSPG samples were digested with nitrous acid (1 μl/1 μg HSPG) using $NaNO_2$ in 1.8 M acetic acid at a final concentration of 0.24 M (*44*). Following a 20 min nitrous acid digestion at room temperature, 5 volumes of absolute ethanol was added to the reaction mixtures, vortexed and centrifuged at 14,000×g for 20 min. The pellets were then dissolved in 1×SDS-PAGE sample buffer for electrophoretic separation and detection of the liberated perlecan core protein. Silver staining of heparitinase/heparinase and nitrous acid digested samples was then employed as described above.

Analysis of Final Perlecan Preparations by Western Blotting—SDS-PAGE was performed as described above and the separated proteins were transferred to nitrocellulose using a Mini transblot electrophoresis transfer cell. Electrotransfer was performed at 100 V for 2 h. Following transfer, membranes were rinsed with water and blocked overnight with 0.15% (w/v) bovine serum albumin, 1% (v/v) normal goat serum, 100 mM Tris-HCl, and 3 mM NaN_3 (pH 7.4). Nitrocellulose membranes probed with fibronectin antibody were blocked in the same solution described above without normal goat serum. Blots were probed with (a) a monoclonal antibody (used at a 1:750 dilution) against perlecan core protein (HK-102), (b) an affinity-purified polyclonal antibody (used at a 1:2,000 dilution) against laminin, (c) a polyclonal antibody (used at a 1:25,000 dilution) against fibronectin (AB1941), and (d) a polyclonal antibody (used at a 1:25,000 dilution) against type IV collagen. The primary antibodies (described above) were diluted with Tris-buffered saline containing 100 mM Tris-HCl, 50 mM NaCl, 0.05% Tween-20, and 3 mM NaN_3 (pH

7.4) (TTBS). Corresponding blots were incubated with primary antibodies for 3 h, washed with TTBS three times (10 min each), followed by a 1 h incubation with the appropriate biotinylated secondary antibodies diluted 1:1,000 with TTBS. The membranes were then rinsed three times (10 min each) with TTBS, probed for 30 min with avidin alkaline phosphatase conjugate (Vectastain), rinsed again (as described above), followed by the addition of an alkaline phosphatase substrate solution (Vectastain). Following color development, the reaction was stopped by flushing the membranes with DD water.

Analysis of Final Perlecan Preparations for Possible DNA Contamination—Possible contamination of the final perlecan preparations by DNA was assessed according to the methods described by Sambrook *et al.* (*45*). Briefly, samples of perlecan (10 and 100 μg) and DNA standards (0-100 ng) in 5 μl of distilled water were mixed with equal volumes of 2 μg/ml of ethidium bromide in 10 mM Tris-HCl with 1 mM EDTA (pH 7.6). The mixtures were spotted on saran wrap on top of a UV transluminator, photographed, and scanned using a densitometer. The amount of DNA in the final perlecan preparation was determined from a calibration curve generated from the optical density of DNA standards.

Use of the Perlecan Product in a Rat Infusion Model—For the rat animal model studies, Aβ (residues 1-40: lot #WM365; Bachem California, Torrance, CA) was initially dissolved in double distilled sterile water at a concentration of 1 mg/ml (stock solution). Fifty microliters of Aβ stock solutions were then transferred with sterile pipettes to microcentrifuge tubes containing either 50 μl of sterile distilled water, or 25 μg of perlecan recently dissolved in 50 μl of distilled sterile water. The Aβ (1-40), and Aβ (1-40)+perlecan solutions were then either frozen at −70°C or used immediately in the animal model (described below).

Twenty male Harlan Sprague-Dawley rats (250-300 g; 3 months old) were anesthetized with pentobarbital (50 mg/kg) and a 27 gauge stainless steel cannula was stereotactically implanted into the hippocampus using bregma as reference point (AP −4.8; ML 3.5; DV 3.0) and secured to the skull by machine screws and dental acrylic. The cannula was connected *via* a 15 cm coil of vinyl tubing to a model 2002 osmotic minipump (Alzet) placed subcutaneously beneath the shoulder blades. The infused solution was contained entirely within the coil of vinyl tubing and separated from water in the pump (dyed blue with food coloring) by a 3 cm air spacer. Successful performance of the pumps was confirmed by measuring movement of the air spacer and blue saline solution following sacrifice. In the present study to demonstrate the effects of isolated EHS perlecan in this animal model, 10 animals received infusion of either Aβ (1-40) only, or Aβ (1-40)+perlecan, directly into hippocampus at a flow rate of 0.5 μl/h for 1 week. A high concentration of Aβ was chosen to maximize possible effects following infusion into brain. The quantity of Aβ peptide infused into brain by the end of 1 week in each animal was approximately 50 μg.

Rats were sacrificed by an overdose of pentobarbital and perfused with 100 ml of saline followed by 150 ml of 4% paraformaldehyde buffered with phosphate (pH 7.4), the brains were removed and postfixed for 48 h, and transferred to PBS for frozen tissue sectioning. Consecutive 25 μm serial sections were cut using a sliding microtome and placed on gelatin-coated slides.

Immunohistochemistry and Staining Techniques—As we previously described (*30*), detection of infused Aβ was monitored using a polyclonal antibody against synthetic Aβ (10D5; gift of Dr. Dale Schenk, Athena Neurosciences) or a monoclonal antibody (6E10; Senetek) which recognizes residues 1-17 of Aβ. Perlecan accumulation was monitored using a polyclonal antibody against the core protein of perlecan (generous gift of Dr. Koji Kimata, Aichi Medical University, Japan). From each animal, 100 consecutive serial sections were cut and stained with cresyl violet to identify the area occupied by the infusion site. Usually, the infusion site spanned 40-60 serial sections. Congo red staining (*46*) and Thioflavin S fluorescence (*47*) were then used on every 10th section spanning through the entire infusion site to determine the extent and consistency of possible fibrillar Aβ amyloid deposition in these animals. The percent of animals containing congophilic deposits (indicative of fibrillar amyloid) in each of the two groups was assessed by blind scoring of tissue sections (scoring of every 10th congo red stained sections through the entire infusion site) using an arbitrary scale of scoring (from 0 to 5) as previously described (*30*). Tissue sections with the anti-Aβ antibodies were pretreated for 3-5 min with 88% formic acid before immunostaining to aid in unmasking hidden antigenic sites as previously reported (*48*). For immunostaining, negative controls consisted of using TBS instead of the primary antibody and/or preabsorption experiments using the primary antibody in the presence of excess antigen (*49*).

RESULTS

Perlecan Purification from the EHS Tumor and Analysis at Each Step of the Isolation Protocol—In order to determine the effectiveness of our perlecan isolation protocol, the presence of perlecan, other PGs and non-PG proteins were monitored at each step of the isolation method (Fig. 1). As shown in Fig. 2, lane 1, the 4 M guanidine-HCl extracts contained two major PGs which were detected by Alcian Blue staining (blue bands). These included a high M_r PG, believed to represent perlecan (at the interface of the resolving gel), and a 220 kDa PG, believed to represent the high density HSPG, previously reported (*33, 36*). The guanidine-HCl extraction protocol recovered >90% of GAGs as determined using an quantitative Alcian blue staining assay (*50*). In addition to the two major PGs (described above), the guanidine-HCl extracts contained many non-PG proteins as shown by Coomassie Blue staining (purple stained bands in Fig. 2, lane 1). The guanidine extracts were then pooled and applied to Sephacryl S-400 columns. Figure 2, lane 2, demonstrates the presence of the two major PGs (detected by Alcian blue) and non-PG proteins (detected by Coomassie Blue) following elution from Sephacryl S-400 columns. Gel filtration chromatography using Sephacryl S-400 columns allowed for both buffer exchange and removal of many (but not all) of the non-PG proteins (compare the Coomassie Blue stained bands in Fig. 2, lane 2, to Fig. 2, lane 1). The void volume fractions from the Sephacryl S-400 columns were then pooled and supplemented with 0.5% Triton X-100 (v/v) and applied to a DEAE-Sephacel column equilibrated with

urea buffer. Subsequent washing of the DEAE column with urea buffer containing Triton X-100 and 0.25 M NaCl removed all of the unbound non-PG proteins, as no Coomassie Blue stained bands were apparent in the last portion of the 0.25 M NaCl eluate (Fig. 2, lane 3). The subsequent 1.0 M NaCl elution from the DEAE-Sephacel column removed the two major PGs present (*i.e.* believed to represent perlecan and the 220 kDa PG) and demonstrated the absence of any non-PG proteins (by lack of positive Coomassie Blue stained bands) (Fig. 2, lane 4). Further elution with 3 M NaCl demonstrated the absence of any residual PGs or non-PG proteins bound to the DEAE column (Fig. 2, lane 5) and indicated that the majority of PGs were removed from the DEAE column using 1.0 M NaCl.

In order to completely separate the 220 kDa PG component from perlecan (shown in Fig. 2, lane 4) gel filtration chromatography using a Sephacryl S-500 was initially employed. It was expected that fractionation using a Sephacryl S-500 would separate perlecan (expected to elute in the void volume) from the 220 kDa PG (expected to elute with a K_{av} of ~0.45). However, under these conditions the 220 kDa PG apparently aggregated and eluted in the void volume, with perlecan eluted shortly thereafter (with a $K_{av}=0.20$). In order to achieve better separation between perlecan and the 220 kDa PG, a variety of different dissociating eluants were tried. These eluants included 4 M guanidine-HCl with 0.1% CHAPS, 7 M urea with 0.1% CHAPS, and 7 M urea with 1% SDS. Regardless of the eluants employed (as described above), the 220 kDa PG was always present in the void volume fractions and was present in most fractions containing perlecan. Separation of perlecan from the aggregating 220 kDa PG was achieved using a Sephacryl S-1000 column. As shown (Fig. 2, lanes 6 and 7; and Fig. 3), this column was effective in separating perlecan from the 220 kDa PG, which under these conditions still aggregated and was primarily found in the void volume fractions with a $K_{av}<0.33$ (Fig. 3). Perlecan was now effectively separated from the 220 kDa PG and was primarily present in fractions with a $K_{av}=0.20$–0.70, with a major peak at $K_{av}=0.40$ (Fig. 3). Perlecan could therefore be successfully isolated without contamination by the 220 kDa PG by pooling fractions with a $K_{av}=0.37$–0.54 (Fig. 2, lane 7, and Fig. 3). The 220 kDa PG (with only slight perlecan contamination) could also be isolated by pooling fractions with a $K_{av}=0.0$–0.37 (Fig. 2, lane 6, and Fig. 3). Any lower molecular weight GAGs observed in the pooled perlecan fractions following a second pass through the Sephacryl S-1000 column (Fig. 2, lane 7; Fig. 3A) were subsequently removed following a third pass through the Sephacryl S-1000 column, yielding a pure perlecan product (see Figs. 4 and 5).

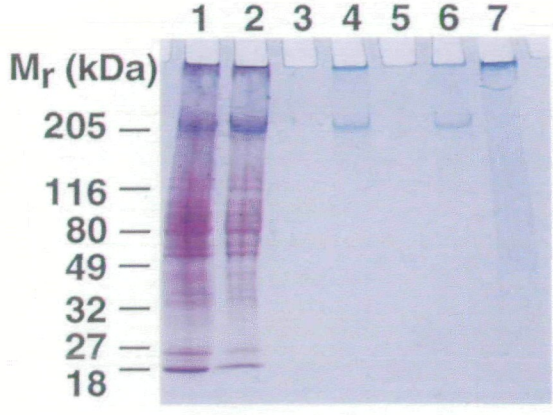

Fig. 2. **Analysis of perlecan purity during each step of the isolation protocol.** This SDS-PAGE gel, stained with Alcian Blue followed by Coomassie Blue, represents an analysis of the purity of perlecan, other PGs, and/or proteins, at each step of the isolation protocol. Alcian blue stained the GAG portion of PGs (blue), whereas Coomassie Blue stained non-PG proteins (purple). Samples were ethanol precipitated prior to SDS-PAGE (see "MATERIALS AND METHODS"). Stds, standard M_r markers. Lane 1: Non-PG proteins (purple bands) and PGs (blue bands) present in the guanidine-HCl extract. Note that the two major PGs present at this step of the isolation protocol are perlecan (at the resolving gel interface) and a 220 kDa PG. Lane 2: Non-PG proteins (purple bands) and PGs (blue bands) eluted from the Sephacryl S-400 column. Lane 3: The last portion of the 0.25 M NaCl wash from the DEAE-Sephacel column demonstrates the complete removal of all non-binding proteins and PGs. Lane 4: PGs (blue bands) eluted from the DEAE-Sephacel column with 5 column volumes of urea buffer containing 1 M NaCl. Note that perlecan (at the resolving gel interface) and the 220 kDa PG are both present. Lane 5: Absence of any appreciable PGs or non-PG proteins from the DEAE-Sephacel column eluted with 5 column volumes of urea buffer containing 3 M NaCl. Lane 6: Presence of the aggregating 220 kDa PG (blue band) in the pooled void volume fraction ($K_{av}=0.0$–0.29) following Sephacryl S-1000 fractionation. Lane 7: Presence of perlecan (blue band at the resolving gel interface) in the included volume fraction ($K_{av}=0.32$–0.54) following a second pass through a Sephacryl S-1000 column. Note no appreciable contamination by the 220 kDa PG or non-PG proteins. Lower M_r GAGs present (blue smear below 205 kDa) were subsequently removed by a third pass through the Sephacryl S-1000 column.

Assessment of Purity of Final Perlecan Preparations by Silver and Alcian Blue Staining—It was important to ensure that the purity of perlecan that was obtained in the final preparations were of the highest quality. For these analyses, a combination of silver staining, Alcian Blue staining and Western blotting were employed (as described below) on aliquots of purified perlecan. In order to determine that no other proteins were present in our final perlecan preparations, silver staining of SDS-PAGE gels were first used. For these studies, 6.25 μg aliquots (determined by Lowry) of perlecan were separated on 4–12% gradient SDS-PAGE gels under reducing conditions and stained with silver (see "MATERIALS AND METHODS"). As shown in Fig. 4A, perlecan was observed at the resolving gel interface, with no other lower M_r bands present. This observation indicated that our final perlecan preparations did not contain any contaminating proteins with a $M_r<$ 700–800 kDa (detectable by silver staining).

This same perlecan preparation was then stained with Alcian Blue (Fig. 4B) to determine possible contamination by other smaller PGs and/or free GAG chains. As shown in Fig. 4B, lane 1, Alcian Blue staining of undigested perlecan demonstrated only one band (indicative of intact perlecan) at the 4–12% gradient SDS-PAGE gel interface. No other Alcian Blue stained bands of lower M_r were observed indicating the absence of any other PGs and/or free GAG chains in our final perlecan preparations. To confirm that the high M_r Alcian Blue stained material (believed to represent perlecan) (Fig. 4B, lane 1) contained heparan sulfate GAGs, digestion with heparinase/heparitinase (specific for heparan sulfate and/or heparin GAGs) was also used. As shown in Fig. 4B, lane 2, following digestion with

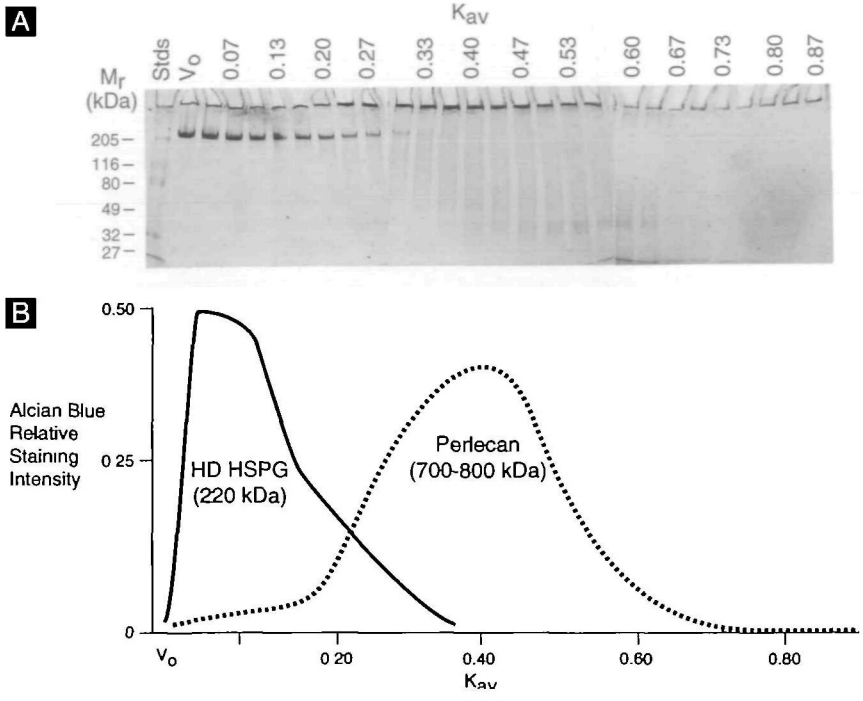

Fig. 3. Alcian Blue stained SDS-PAGE gels of fractions obtained from a second pass through a Sephacryl S-1000 column. A: Three adjacent Alcian Blue stained SDS-PAGE gels illustrating the presence of perlecan (band at the gel interface) and the aggregating 220 kDa PG in fractions obtained from the Sephacryl S-1000 column. Note that perlecan can be separated from the 220 kDa PG by pooling fraction with $K_{av} > 0.37$. B: Relative Alcian Blue staining intensities of perlecan (700-800 kDa) and the 220 kDa PG in fractions obtained from the Sephacryl S-1000 column.

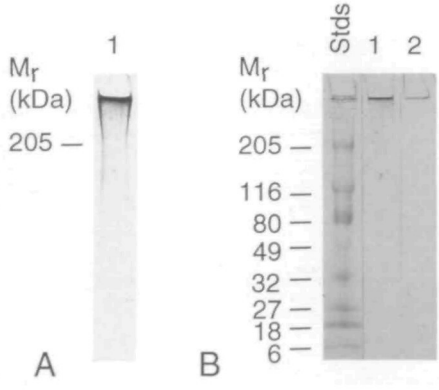

Fig. 4. Silver and Alcian Blue stained SDS-PAGE gels to assess purity of final perlecan preparations. A: Silver staining of final perlecan preparation (pooled fractions K_{av} 0.29-0.54 from a third pass through a Sephacryl S-1000 column) illustrating the presence of perlecan (band at the gel interface). Note absence of 220 kDa PG and any other proteins. B: Alcian Blue stained gel demonstrating the presence of undigested perlecan (lane 1, blue band at the gel interface), and perlecan following digestion with heparinase/heparitinase enzymes (lane 2). Note a decrease in Alcian Blue staining of band at the gel interface in lane 2 in comparison to lane 1 demonstrating successful digestion of heparan sulfate GAGs. Stds, standard M_r markers.

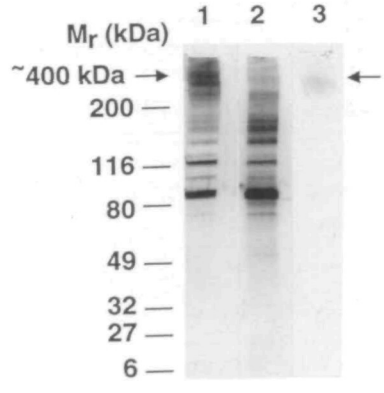

Fig. 5. Silver staining of final perlecan product following heparitinase/heparinase and nitrous acid digestions. Silver staining of final perlecan preparations to ensure intact perlecan core protein and absence of any contaminating proteins/PGs. Lanes 1 and 2 were run on the same SDS-PAGE gel, whereas lane 3 was run on a separate gel. Lane 1: Silver staining of the final perlecan preparation following heparitinase/heparinase digestion. Note the doublet at ~400 kDa (arrow) characteristic of intact perlecan core protein. All of the other silver stained bands are due to the presence of the heparitinase/heparinase enzymes (compare to lane 2). Lane 2: Silver staining of the heparitinase/heparinase enzymes only. Compare to lane 1. Lane 3: Silver staining of the final perlecan preparation following nitrous acid treatment. A band at ~400 kDa is present (arrow) indicative of intact perlecan core protein. Note that no other silver stained bands are present.

heparitinase/heparinase a marked reduction in Alcian Blue staining was observed (compare Fig. 4B, lane 2 to lane 1). This indicated that the putative perlecan band (shown in Fig. 4B, lane 1) contained heparan sulfate GAG chains.

In order to demonstrate that the Alcian blue and silver stained bands observed at the resolving gel interface in Fig. 4A (lane 1) and 4B (lane 1) were in fact intact perlecan, and that no other proteins or contaminating PGs were present in the final perlecan preparations, heparitinase/heparinase and nitrous acid digested samples of the final perlecan product were also stained with silver (Fig. 5). As shown in Fig. 5, lane 1, (arrow) a discrete doublet, characteristic of the perlecan core protein, was observed at ~400 kDa by silver staining following heparitinase/heparinase digestion. Silver staining of only the heparitinase/heparinase enzymes employed (in the absence of perlecan) (Fig. 5, lane 2) did not show the characteristic ~400 kDa doublet observed in Fig. 5, lane 1. Nitrous acid digestion of the final perlecan product followed by silver staining also yielded

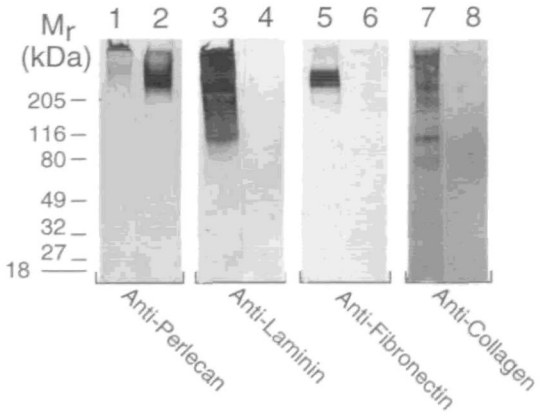

Fig. 6. **Western blot analysis of final perlecan preparations.** Western blot analysis of final perlecan preparations (lanes 1, 2, 4, 6, and 8) probed with a antibodies to perlecan core protein (anti-HK-102; lanes 1 and 2), laminin (lanes 3 and 4), fibronectin (lanes 5 and 6), and collagen type IV (lanes 7 and 8). Lane 1: Undigested perlecan probed with a perlecan core protein monoclonal antibody (HK-102). Perlecan is observed at the gel interface. Lane 2: Heparinase/heparitinase digestion of perlecan, probed with the perlecan core protein antibody (HK-102), reveals a characteristic doublet at 400 and 360 kDa, indicative of intact perlecan core protein. Lane 3: Laminin probed with a polyclonal antibody against laminin. Positive control for lane 4. Bands for laminin detected by the laminin antibody consist of 400 and 280 kDa subunits dissociated from each other upon reduction. Lane 4: Final perlecan preparation probed with a laminin antibody. No contamination by laminin was found. Lane 5: Fibronectin probed with a polyclonal antibody against fibronectin. Positive control for lane 6. Bands for fibronectin detected by the fibronectin antibody consist of a dimer derived 250 kDa dissociated from each other upon reduction. Lane 6: Final perlecan preparation probed with a fibronectin antibody. No contamination by fibronectin was found. Lane 7: Type IV collagen probed with a polyclonal antibody against type IV collagen. Positive control for lane 8. Lane 8: Final perlecan preparation probed with a type IV collagen antibody. No contamination by type IV collagen was found.

similar results (Fig. 5, lane 3). A characteristic ~400 kDa band indicative of the intact perlecan core protein was observed on silver staining following nitrous acid pretreatment (Fig. 5, lane 3). These studies indicated that no other protein or PG bands were present in the final perlecan preparations, even following liberation of the perlecan core protein by heparitinase/heparinase or nitrous acid.

Assessment of Purity of Final Perlecan Preparations by Western Blotting—In order to further demonstrate and confirm the presence of perlecan, and the absence of other basement membrane components produced by the EHS tumor (*i.e.* laminin, fibronectin and type IV collagen) in the final perlecan preparations, Western blotting with specific antibodies was employed. Figure 6, lane 1 is a Western blot of our final perlecan preparation following separation on a 4-12% gradient SDS-PAGE (under non-reducing conditions) probed with a monoclonal antibody (HK-102) against perlecan core protein. As shown, perlecan immunoreactivity was apparent at the resolving gel interface, proving that the positive silver (Fig. 4A) and Alcian Blue (Fig. 4B, lane 1) staining observed at the resolving gel interface previously, was in fact intact perlecan core protein. Following heparinase/heparitinase digestion (Fig. 6, lane 2) the perlecan monoclonal antibody detected a doublet at 400 and 360 kDa, characteristic and indicative of intact perlecan core protein (*5, 33, 34*).

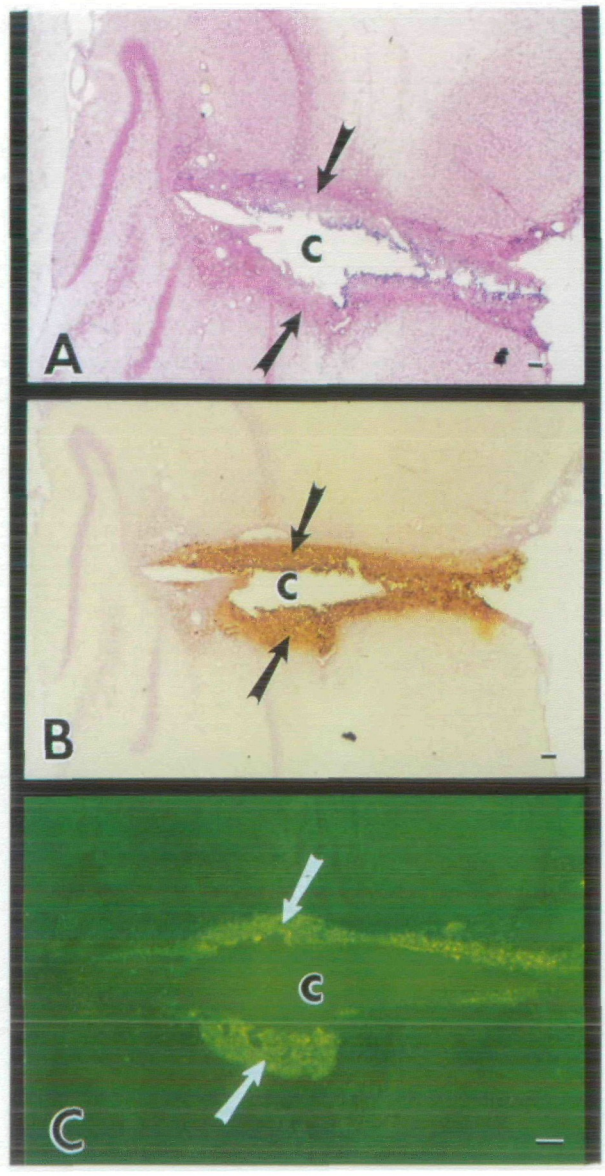

Fig. 7. **Infusion of the perlecan product plus Aβ (1-40) into rodent hippocampus for a reliable animal model of Aβ amyloid deposition/persistence.** A: 1 week infusion of Aβ (1-40)+perlecan into rodent hippocampus. The cannula tract (c) is observed penetrating into cortex. The infusate has penetrated into brain parenchyma and is present on either side of the cannula tract (arrows). Cresyl violet staining. Bar=100 μm. B: Serial section from A demonstrating the extent of Aβ deposition (arrows) penetrating into brain parenchyma, as detected using a polyclonal antibody (10D5) against Aβ. Bar=100 μm. C: Adjacent section from B demonstrating positive Thioflavin S fluorescence (arrows) (indicative of amyloid) at the infusion site. Note that the amyloid site corresponds precisely to the area occupied by Aβ plus perlecan (not shown). Bar=100 μm.

The purity of the final perlecan preparations were further confirmed by Western blotting and probing with a series of antibodies directed against other basement membrane components known to be produced by the EHS tumor and known to bind to perlecan. As shown in Fig. 6, lane 3, although 1 μg of EHS laminin was strongly stained on Western blot when probed with an anti-laminin antibody

(positive control for Fig. 6, lane 4), no positive immunostaining for laminin was observed in 6.25 μg of our final perlecan preparation (Fig. 6, lane 4). Similarly, although 1 μg of fibronectin stained strongly on Western blot when probed with an anti-fibronectin antibody (Fig. 6, lane 5), no positive immunostaining for fibronectin was observed in 6.25 μg of our final perlecan preparation. Furthermore, although 1 μg of type IV collagen stained strongly on Western blot when probed with an anti-type IV collagen antibody (Fig. 6, lane 7), no positive immunostaining for type IV collagen was observed in 6.25 μg of our final perlecan preparation (Fig. 6, lane 8). These latter studies indicated that only perlecan, and no other basement membrane components produced by the EHS tumor, was present in the final perlecan preparations.

The final perlecan preparation was also analyzed for possible contamination by DNA. One-tenth percent (10 ng of DNA was detected in 10 μg of perlecan) of DNA was found in our final perlecan preparations indicating almost negligible contamination.

Use of the Perlecan Product in a Rat Infusion Model—High quality perlecan was essential to establish a consistent and reproducible animal model to study the effects of fibrillar Aβ amyloid deposits in rodent brain (*30*). Following a 1-week infusion of Aβ (1-40) alone, or Aβ (1-40)+ perlecan into rodent hippocampus, it was evident that differences existed in the extent and percent of animals with Aβ fibrillar amyloid deposits. One hundred percent (10 of 10) of animals infused with Aβ (1-40)+perlecan for 1 week demonstrated Congo red and Thioflavin S-positive deposits (indicative of amyloid) at the infusion site (Fig. 7). In comparison only 60% (6 of 10) of animals infused with Aβ (1-40) alone demonstrated Congo red or Thioflavin S-positive deposits at the infusion site. The Congo red (not shown) and Thioflavin S (Fig. 7B) positive deposits in the Aβ+perlecan group corresponded on adjacent serial sections to precisely those areas containing both Aβ (Fig. 7B) and perlecan (not shown), as previously demonstrated (*30*). This *in vivo* study therefore demonstrated that consistent fibrillar Aβ deposition and persistence could be obtained with the use of a high quality perlecan product (in the presence of Aβ 1-40).

DISCUSSION

The present investigation described a novel and relatively rapid method for isolation of perlecan from the EHS tumor. The protocol is unique in that the method did not require cesium chloride density gradient centrifugation, as has been previously described by all investigators (*5, 33-36*). The perlecan product obtained by our protocol was of high quality and did not contain any contaminants produced by the EHS tumor, including other basement membrane components (*i.e.* laminin, fibronectin, and type IV collagen) (assessed by silver staining and Western blotting with specific antibodies) and/or free GAG chains (assessed by Alcian Blue staining). In addition, the final perlecan preparation was found to be essentially free of potential contaminating DNA. The presence of contaminating basement membrane proteins (especially laminin) in perlecan preparations has posed a major problem in the past, and it is essential that the perlecan used for *in vitro* and/or *in vivo* studies be thoroughly analyzed for components which can potentially interact with perlecan during the isolation procedure. In the present study, a detailed perlecan isolation procedure employed for consistent production of high quality perlecan was described. In addition, the quality control steps employed to ensure the production of an essentially clean perlecan product was emphasized.

Our Perlecan Isolation Protocol in Comparison to Previously Described Methods—Isolation of perlecan has been previously described by several investigators (*5, 33-36*). In one previous study (*33*), perlecan was isolated from EHS tumor initially using nondenaturing conditions (extraction with buffered saline), followed by denaturing conditions (extraction with urea). The urea extracts were then processed utilizing DEAE-Sephacel chromatography, followed by ultracentrifugation, dialysis, gel filtration chromatography (Sepharose CL-4B), exhaustive dialysis, and lyophilization. One of the problems noted by the authors in this procedure of perlecan purification (*33*) was the contaminating presence of laminin and other proteins, believed to be inherently bound to perlecan by disulfide-dependent association, which could only be removed by disulfide bond reduction (using dithiothreitol) followed by ion exchange chromatography. In another study, increased purity was achieved when the protocol was modified by pre-extracting the tumor tissue with 3.4 M NaCl before extraction with 6 M urea (*34*). However, this latter method resulted in occasional partial degradation of the large 400 kDa perlecan core protein (*35*).

Extraction of EHS tumor with chaotrophic agents such as 4 M guanidine-HCl allowed for rapid and efficient extraction of PGs (*2, 5, 51*) and was the method of choice in our protocol. The denaturing conditions and the use of protease inhibitors in the extraction solution protected the integrity of the large perlecan core protein (see Fig. 2, lane 1), which was susceptible to proteolysis (*35*). We calculated that >90% of GAGs (as determined by an Alcian Blue assay) (*50*) were extracted from EHS tumor tissue using 4 M guanidine-HCl containing 0.5% CHAPS and protease inhibitors (not shown).

In addition to the presence of PGs, the guanidine extracts contained large amounts of non-PG proteins which may potentially interfere with subsequent ion-exchange chromatography. Gel filtration chromatography using a Sephacryl S-400 column allowed for both buffer exchange (7 M urea with detergent and protease inhibitors) and the removal of a large number of small molecular weight proteins (compare Fig. 2, lane 2 to lane 1) which could not be accomplished by a much longer dialysis process, as some have previously described (*2, 33*). The guanidine-HCl extract from 50 g of EHS tumor (~400 ml) was exchanged into 7 M urea buffer (described above) in less than one day, requiring less than 3 liters of buffer.

Yield of Perlecan Using the Described Protocol—The final yield of perlecan using the described method was approximately 10-12.5 μg per gram of EHS tumor wet weight, as measured by protein determination (*52*). Although clonal differences in EHS tumor may result in variations in GAG chain length (*35*), a protein to GAG ratio of approximately 1:1 in our purified perlecan product was calculated (not shown). This is similar to GAG:protein ratios of the low density HSPG (*i.e.* perlecan) previously described by Fujiwara *et al.* (*36*). Additionally, the total GAG content present in the EHS tumor tissue determined

by using an Alcian Blue assay (*50*), was approximately 0.90 mg of GAGs per gram wet weight of EHS tumor (not shown). This is comparable to the 0.75 mg of GAGs per gram wet weight of EHS tumor previously reported by Fujiwara *et al.* (*36*). Following S-1000 fractionation, only 20% (150-180 μg per gram tumor wet weight) of the calculated GAG content was found to be attributed to perlecan [consistent with the study by Dziadek *et al.* (*4*)], whereas most of the remaining GAG content was present in the 220 kDa PG (not shown). Based on these determinations, the final perlecan yield using the described protocol from starting EHS tumor tissue was approximately 10%. Two hundred grams of EHS tumor was routinely found to yield approximately 4-5 mg of perlecan.

Identification of an Aggregating 220 kDa PG Which Can Be Separated from Perlecan by Gel Filtration Chromatography—Earlier studies have demonstrated two major PGs to be produced by the EHS tumor (*2, 4, 33, 34, 36*). Based on cesium chloride density gradient centrifugation they were designated as a high density HSPG ($\geq$1.65 g/ml) and a low density HSPG (1.38 g/ml) (*2, 33, 34*). The low density HSPG was later found to be analogous to perlecan, whereas the high density HSPG had an electrophoretic mobility of 200-400 kDa (*36*), and was postulated to represent either a breakdown product of the low density HSPG (*i.e.* perlecan) (*33, 34*), or an independent PG derived from a separate gene product (*2, 35*). Although these latter possibilities have not been fully resolved, the high density HSPG previously identified by cesium chloride centrifugation is most likely identical to the 220 kDa PG that we described. This 220 kDa PG, like the previously described high density HSPG (*2, 33, 36*), was found to be the major PG extractable by saline and contained heparan sulfate GAG chains as demonstrated by degradation with heparinase/heparitinase digestion (Castillo and Snow, unpublished observations). On occasion, some of the EHS tumor isolations produced a 220 kDa PG which was also sensitive to chondroitinase ABC (Snow and Castillo, unpublished observations), therefore producing a hybrid PG (containing both heparan sulfate and chondroitin/dermatan sulfate GAGs), as was previously reported (*2*).

One of the unique findings in the present investigation was that under certain chromatographic conditions the 220 kDa PG self-aggregated, which enabled it to be separated from perlecan (which did not aggregate under similar conditions). These differences in aggregating properties allowed us to develop a novel method to purify perlecan without contamination by the 220 kDa PG, using Sephacryl S-1000 chromatography. The self-aggregating property of the 220 kDa PG appeared not to involve any other proteins as determined by analysis of SDS-PAGE gels by silver staining (not shown). The 220 kDa PG eluted from the Sephacryl S-1000 in the void volume (see Fig. 3) indicating that this PG formed large aggregates of $\gg$100,000 kDa, which occurred even under dissociating conditions. This result differed from the lack of self-aggregation of a 220 kDa PG (referred to as the high density PG) eluted on a Sepharose CL-4B column under dissociating conditions as reported by Hassell *et al.* (*33*). This apparent discrepancy may be explained by (a) prior use of cesium chloride centrifugation which was not used in our protocol, and/or (b) less than generous use of protease inhibitors in the earlier study. Each of these possibilities could have led to the alteration or degradation of epitopes important for self-aggregation of the 220 kDa PG. We are currently determining the amino acid sequence and possible core protein size(s) of the 220 kDa PG to assess whether this particular PG is distinct from perlecan and is derived from a separate gene product.

Use of the Perlecan Product in a Rat Infusion Model to Study the Effects of Fibrillar Aβ Amyloid in Brain—Of all the different classes of PGs that have now been identified in the characteristic lesions of AD (including heparan sulfate, dermatan sulfate, chondroitin sulfate and keratan sulfate) (*53-56*), only HSPGs have been found to be immunolocalized to all three major lesions (*i.e.* neuritic plaques, neurofibrillary tangles, and cerebrovascular amyloid deposits). In addition, previous studies have demonstrated HSPGs to be an invariant component of all amyloid deposits (both systemically and in the central nervous system), regardless of the amino acid sequence of the specific amyloid protein involved (*25, 57, 58*). The specific HSPG, perlecan has been identified in virtually every case in which amyloid-associated PGs have been characterized (*53, 59-61*) and is believed to play an important role in amyloid fibrillogenesis, deposition, accumulation, and persistence (*25*).

The consistent high quality of isolated perlecan has led to its use in the development of an animal model in which to study the effects of fibrillar Aβ amyloid deposits in brain. In initial studies (*30*) continuous one week infusion of Aβ (1-40) + perlecan demonstrated 100% of animals with Congo red and Thioflavin S-positive deposits at the infusion site. Significant increases in fibrillar Aβ amyloid deposits in brain was observed in animals infused with Aβ + perlecan, in comparison to animals infused with Aβ alone. In addition, only 50% of the animals infused with Aβ (1-40), in the absence of perlecan, demonstrated Aβ amyloid deposits. This initial study suggested that perlecan contributed to the consistency and persistence of fibrillar Aβ amyloid in brain. Similar results were obtained in the present study using perlecan product derived from the described protocol. Consistent (100%) fibrillar Aβ amyloid deposition and persistence in rat brain was observed in 100% (10 of 10) animals infused with perlecan + Aβ, compared to only 60% (6 of 10) following infusions of Aβ alone. Our studies utilizing this animal model suggest that the final purity of the perlecan preparation is essential for continued reliability of fibrillar Aβ amyloid deposition and persistence in brain. Contaminating proteins such as laminin, fibronectin and type IV collagen, which are produced by the EHS tumor and which normally associate with perlecan can cause unexpected variability in the animal model (Snow, Cummings, and Castillo, unpublished observations). Other investigators who attempt to replicate our findings with the described rodent model must ensure that the perlecan used is of high quality and free of contaminating proteins. Even the presence of free GAG chains (*i.e.* heparan sulfate) in perlecan preparations will compete with perlecan for binding sites on Aβ and will disrupt the consistency of this model (Snow and Castillo, unpublished observations).

Use of High Quality Perlecan for Development of In Vitro Assays—Although the use of high quality perlecan led to the development of an *in vivo* model to study the effects of Aβ amyloid in brain, other uses of contamination-free perlecan will augment future development of *in vitro*

assays. Binding assays to determine the interaction of perlecan with other proteins and/or PGs will prove unreliable if contaminants are present in the perlecan preparations. These contaminants may serve to either mask perlecan binding sites to a potential candidate macromolecule, or have the ability to bind to the candidate molecule itself. Even the uncertainty of perlecan purity can pose a problem in the final interpretation of the results. Stringent quality control testing that should be employed for each and every perlecan isolation will hopefully ensure reliable interpretation of experimental data obtained in the future for both *in vivo* and *in vitro* biological applications.

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