Endothelial Cells Interact with the Core Protein of Basement Membrane Perlecan through β1 and β3 Integrins: An Adhesion Modulated by Glycosaminoglycan

Kimiko Hayashi, Joseph A. Madri,* and Peter D. Yurchenco

Department of Pathology, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854; and *Department of Pathology, Yale University School of Medicine, New Haven, Connecticut 06510

Abstract. Aortic endothelial cells adhere to the core protein of murine perlecan, a heparan sulfate proteoglycan present in endothelial basement membrane. We found that cell adhesion was partially inhibited by β1 integrin-specific mAb and almost completely blocked by a mixture of β1 and αvβ3 antibodies. Furthermore, adhesion was partially inhibited by a synthetic peptide containing the perlecan domain III sequence LPASFRGDKVTSY (c-RGD) as well as by GRGDSP, but not by GRGESP. Both antibodies contributed to the inhibition of cell adhesion to immobilized c-RGD whereas only β1-specific antibody blocked residual cell adhesion to proteoglycan core in the presence of maximally inhibiting concentrations of soluble RGD peptide. A fraction of endothelial surface-labeled detergent lysate bound to a core affinity column and 147-, 116-, and 85-kD proteins were eluted with NaCl and EDTA. Polyclonal anti-β1 and anti-β3 integrin antibodies immunoprecipitated 116/147 and 85/147 kD surface-labeled complexes, respectively. Cell adhesion to perlecan was low compared to perlecan core, and cell adhesion to core, but not to immobilized c-RGD, was selectively inhibited by soluble heparin and heparan sulfates. This inhibition by heparin was also observed with laminin and fibronectin and, in the case of perlecan, was found to be independent of heparin binding to substrate. These data support the hypothesis that endothelial cells interact with the core protein of perlecan through β1 and β3 integrins, that this binding is partially RGD-independent, and that this interaction is selectively sensitive to a cell-mediated effect of heparin/heparan sulfates which may act as regulatory ligands.

VASCULAR endothelial cells are polar cells that form a nonthrombogenic barrier and which, after focal injury, migrate and divide to close the defect. These cells produce and reside upon basement membranes which influence cell adhesion, shape, migration, and differentiation. One of the components of many basement membranes (Kato et al., 1988) is perlecan, a low density basement membrane heparan sulfate proteoglycan (HSPG)[1] (Hassell et al., 1985; Paulsson et al., 1987*a*; Noonan et al., 1991). The core protein of perlecan is a multidomain structure with regions homologous to laminin, the LDL receptor and N-CAM (Noonan et al., 1988, 1991). By EM, the core is visualized as a tandem linear array of five to six globules with typically three heparan sulfate chains extending from one end (Paulsson et al., 1987*a*; Yurchenco et al., 1987). Depending upon tissue location or state of differentiation, the core can possess both high and undersulfated chains (Pejler et al., 1987), long or very short chains (Mohan and Spiro, 1991) and tissue cultured colonic carcinoma cells have been shown to secrete both HSPG as well as core free of any attached glycosaminoglycan chains (Iozzo and Hassell, 1989).

The multidomain structure suggests that perlecan integrates a variety of functions. While many of these functions have yet to be elucidated, there is evidence that the carboxyl-terminal domain mediates self assembly in vitro with formation of dimers and trimers (Yurchenco et al., 1987) and the core binds to fibronectin (Heremans et al., 1990). The polyanionic heparan sulfate chains, attached to the NH_2-terminal domain I of the core, are important in impeding the passage of cationic macromolecules across the glomerular basement membrane (Farquhar, 1981) and may also play a role in hemostasis and the immobilization of growth factors. A variety of cells such as hepatocytes interact with this macromolecule binding through 80 and 26–38 kD surface proteins (Clément et al., 1989; Clément and Yamada, 1990). Inspection of the published sequence of the proteoglycan core reveals that the integrin-binding sequence arg-gly-asp (RGD) is present in a globular region present in domain III of mouse perlecan, a sequence homologous to domain IVb of the laminin A chain (Noonan et al., 1988, 1991): this sequence suggests that a subclass of cell surface integrins plays a role in cell interactions and signal transduction.

1. *Abbreviations used in this paper*: BAEC, bovine aortic endothelial cells; EHS, Engelbreth-Holm-Swarm; HSPG, heparan sulfate proteoglycan; HSPG-core, core protein of heparan sulfate proteoglycan.

In this study we have examined the interaction of primary explant bovine aortic endothelial cells with perlecan with respect to adhesion rate, morphology, effect of glycosaminoglycan side chains and the cell surface proteins participating in adhesion. We report that this interaction is dependent upon the presence of heparan sulfate, and that adhesion is mediated by two or more integrins that recognize an RGD-sequence as well as one or more other regions.

Materials and Methods

Macromolecules

Perlecan (low density, high molecular weight basement membrane HSPG) was purified from lathyritic mouse Engelbreth-Holm-Swarm (EHS) tumor by urea extraction, DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) ion exchange chromatography, cesium chloride gradient density centrifugation, and Sephacryl S500 (Pharmacia Fine Chemicals) gel filtration as previously described in detail (Yurchenco et al., 1987). EDTA extracts of laminin from EHS tumor were purified by gel filtration and ion exchange chromatography as described (Schittny and Yurchenco, 1990) and type IV collagen was isolated from the same tumor by guanidine-HCl extraction, NaCl salt fractionation, and ion exchange chromatography on DEAE-cellulose (Yurchenco and Furthmayr, 1984). Bovine fibronectin was obtained from plasma by gelatin-Sepharose chromatography (Ruoslahti et al., 1981) and rat fibronectin (used for immunoblotting) was purchased from Sigma Chemical Co. (St. Louis, MO). Mouse vitronectin was obtained from Telios Pharmaceuticals, Inc. (San Diego, CA) and type I collagen from Collagen Corp. (Palo Alto, CA). Mouse EHS entactin (nidogen) was purified by the method of Paulsson et al. (1987*b*). The core protein of perlecan was prepared as follows: purified HSPG was dialyzed against 50 mM Tris, pH 7.4, containing 1 mM PMSF in the cold. The HSPG (1 mg/ml) was then incubated with 1.5 conventional units/ml (0.0088 IU/ml) heparitinase (Seikagaku Kogyo, Tokyo, Japan) in the above buffer containing 5 mM $CaCl_2$, 0.5 mM diisopropyl fluorophosphate for 20 h at 37°C. The reaction was stopped with 5 mM EDTA. Heparan sulfate was released from intact proteoglycan by incubating the sample in 0.05 M NaOH/1M $NaBH_4$ at 45°C for 48 h (Yurchenco et al., 1987). The free heparan sulfate was dialyzed exhaustively against water and lyophilized. Bovine kidney heparan sulfate (Seikagaku Kogyo), pig intestinal heparin (Calbiochem Corp., La Jolla, CA), de-*N*-sulfated heparin (Sigma Chemical Co.), chondroitin sulfates A, B, and C (Sigma Chemical Co.), and dextran sulfate (5 kD; Sigma Chemical Co.) were obtained from commercial sources.

Synthetic Peptides

Soluble synthetic peptides, GRGDSP and GREDSP were purchased from Telios Pharmaceuticals, Inc. A synthetic peptide containing the RGD sequence in perlecan (EGF repeat of domain III) was prepared by the protein chemistry facility in the Department of Molecular Biophysics and Biochemistry (Dr. Jim Elliot) at Yale University School of Medicine (New Haven, CT). This peptide was composed of 14 amino acids with the sequence *N*-cys-leu-pro-ala-ser-phe-arg-gly-asp-lys-val-thr-ser-tyr-C. After a cysteine substitution for serine to permit coupling to protein, the 13 residues are those of perlecan in domain III, from residues 993–1005 as deduced from sequencing of overlapping cDNA clones (Noonan et al., 1991). Purity and composition were evaluated by C18 reverse phase HPLC (single peak), by amino acid analysis, and by mass spectrometry: the latter revealed a major peak with a molecular mass of 1,543.0, nearly identical to that predicted (1,542.8). The peptide was found to be readily and completely soluble in PBS. An aliquot of this peptide was coupled to BSA via maleimidobenzoyl-sulfosuccinimido (Pierce Chemical Co., Rockford, IL). 5 mg of peptide was added to maleimidobenzoylsulfosuccinimido-activated BSA, and the formed covalent complex was incubated overnight at 4°C, dialyzed in PBS, and stored at −120°C.

Antibodies

Polyclonal antiserum was prepared against mouse perlecan by immunizing a New Zealand white rabbit with purified perlecan in complete Freund's adjuvant. Specific antibody was isolated by affinity purification of serum on an immobilized core protein column (coupling method described below). This antibody was found to work well in immunoblots. mAb (HK102) specific for core protein of perlecan (low density HSPG) was a kind gift from Dr. Koji Kimata (Aichi University, Japan) and was used for immunoelectron microscopy. Polyclonal antiserum was similarly generated against the long arm of mouse laminin (fragments E3 and E8): from this an A chain–specific reagent was prepared by fragment E3 affinity chromatography followed by cross-absorption against fragments E8, E4, and E1′. Rabbit anti-mouse fibronectin was purchased from Telios Pharmaceuticals, Inc. Blocking mAb AIIB2 (Hall et al., 1990), reactive against the ectodomain of β1 integrin and capable of inhibiting β1-mediated cell attachment, was a kind gift of Dr. Caroline Damsky (University of California, San Francisco, CA). Blocking mAb antibody LM609 (Cheresh and Spiro, 1987), which recognizes the ectodomains of $\alpha v\beta 3$ integrin, was a gift of Dr. David Cherish (Scripps Clinic, San Diego, CA). The two blocking integrin antibodies were obtained in ascites fluid with antibody representing 16 and 19% of total protein (AIIB2 and LM609), respectively. Sufficient AIIB2 was obtained to permit purification by ammonium sulfate precipitation for selective experiments. Polyclonal rabbit antibodies specific for β-1 and for β-3 integrins were prepared from the COOH-terminal 36 residues (Bason et al., 1990).

ELISA Competition Assay

96-well Linbro type EIA microtitration plates (Flow Labs, Inc., McLean, VA) were coated with 0.1 ml per well antigen (1 μg/ml) in 50 mM sodium carbonate/bicarbonate buffer, pH 9.6. Subsequent incubation and wash steps were carried out in PBS containing 0.6% Triton X-100 and 0.1% BSA. Wells were incubated with antibody in the presence of serial dilutions of competing antigen, washed, and then incubated with a 1:6,000 dilution of protein A conjugated to HRP (Sigma Chemical Co.). Color was developed with 150 ml/well of 0.1 mM *o*-phenylenediamine in 50 mM citric acid/100 mM sodium phosphate buffer containing 0.04% H_2O_2 followed by the addition of 50 μl of 2 M sulfuric acid. Absorbance was read at 490 nm.

Cells and Cell Attachment Assay

Bovine aortic endothelial cells (BAEC) used for this study were prepared from calf aortas as previously described (Madri et al., 1980), and cells were used between passages 8 and 11. Cell attachment to substrate-bound macromolecules was assayed as follows: confluent BAEC were trypsinized, washed, and plated (2.0×10^4) on bacteriological-grade polystyrene 96-well plates (Costar Corp., Cambridge, MA) coated with 20 μg/ml perlecan or perlecan-core, 5 μg/ml laminin, 15 μg/ml fibronectin, 10 μg/ml type IV collagen, and 10 μg/ml BSA. The fraction of bound substrate, on a mass basis, was found to be nearly identical when wells were incubated at these concentrations as measured with radioiodinated substrate (0.02 $\mu g/cm^2$ perlecan and perlecan core, 0.02 $\mu g/cm^2$ laminin, 0.02 $\mu g/cm^2$ fibronectin, and 0.015 $\mu g/cm^2$ type IV collagen). Unless otherwise indicated, cells were incubated at 37°C in serum-free Dulbecco's modified Eagle medium (DME) for 2 h. To inhibit protein synthesis, cells were exposed to 2.5 μg/ml cycloheximide 1.5 h before trypsinization until the time of trypsinization. Under this condition >80% of protein synthesis was inhibited up to 8 h after replating on substrate (as measured by TCA precipitation of cell-incorporation of [^{3}H]leucine) while viability remained >95% for the first 4 h (trypan blue dye exclusion) with ~5% detachment at 4 h. A variation of the inhibition protocol was used in some experiments (Figs. 1 and 3) in which cycloheximide was incubated throughout the adhesion assay (protein synthesis was inhibited by 94% between 0 and 2 h as calculated from the difference in TCA-precipitated [^{35}S]methionine at 2 h and at 0 h in the presence of cycloheximide divided by the difference precipitated in the absence of cycloheximide). No differences in the effect on cell adhesion were noted with these treatments. After the incubation, dishes were washed three times with PBS and either stained with toluidine blue for photography or quantitatively assayed for cell adhesion (see below). Amounts of protein substrates bound to the wells were determined by adding a trace amount of ^{125}I-labeled proteins to unlabeled substrate solution before the experiment. Cell number was determined by a colorimetric acid phosphatase assay performed in 96-well tissue culture plates. Phosphatase in bound cells was measured by incubating the wells with enzyme for 1 h with 10 mM *p*-nitrophenyl phosphatase (Sigma Chemical Co., number 104-0 phosphatase substrate), 0.1% Triton X-100, 0.1 M sodium acetate, pH 5.5 (Connolly et al., 1986; Basson et al., 1990). Color was developed by adding 1 N NaOH and absorbance (405 nm) was converted to the percentage of the cells attached to the matrix using standard curve of the cell number.

Radioiodination

(*a*) BAEC cell surface proteins. 2 ml suspension of confluent BAEC (1 ×

10^7 cell/ml) was labeled with 2 mCi of $Na^{125}I$ by lactoperoxidase-catalyzed iodination (Lew et al., 1986) for 40 min on ice using 200 μg enzyme and 0.012% hydrogen peroxide. 150 μl of 0.2 M NaI was added and the cells were washed extensively and processed for surface membrane extraction. Cells were extracted 1 h at 4°C with extraction buffer containing 200 mM octylglucoside (Sigma Chemical Co.), 2 mM PMSF, 10 mM sodium phosphate, pH 7.4, in 127 mM NaCl (PBS). (*b*) Heparin. Tyramine-conjugated heparin, prepared according to the method of Lee et al. (1991), was kindly provided by Dr. Arthur Lander (Massachusetts Institute of Technology, Cambridge, MA). A glass tube was coated with ~10 μg of iodogen (Pierce Chemical Co.). 50 μl of 0.25 M sodium phosphate buffer (pH 7.5) containing several micrograms of conjugated heparin was mixed with 4.5 mCi $Na^{125}I$, incubated 15 min at room temperature, desalted on a Sephadex G25 column, and fractionated on a Sepharose CL6B (50 × 0.6 cm) column. (*c*) Protein A. 5 mg of *Staphylococcus aureus* protein A (Pharmacia Fine Chemicals) was labeled with 5 mCl of $Na^{125}I$ by lactoperoxidase-catalyzed iodination at room temperature using 25–50 μg enzyme and 50 μl of 0.003% hydrogen peroxide for 15–20 min. Labeled protein was affinity purified on a rabbit IgG affinity column.

Affinity Chromatography

HSPG-core affinity columns were prepared by coupling 1 mg of proteoglycan to 2 ml Sepharose CL-4B beads (Pharmacia Fine Chemicals) after cyanogen bromide activation (0.05 g/ml beads). Cell extracts were centrifuged at 1,000 *g* for 10 min. Supernatant was subjected to the column preequilibrated with column buffer (50 mM octylglucoside, 1 mM PMSF, PBS, pH 7.4), and were eluted with a linear gradient of 0.15–1 M NaCl in presence of magnesium chloride, calcium chloride, or EDTA followed by 0.1 M glycine and 8 M urea. A control column was made by coupling BSA (Sigma Chemical Co.) with Sepharose 4B. Fractions eluted from HSPG-core affinity chromatography were analyzed for radioactivity (1-μl aliquots counted in a gamma counter LKB-Wallac model 1271, Turko, Finland), pooled, and dialyzed against PBS containing 1 mM PMSF, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Sample was lyophilized and solubilized for the SDS-PAGE. The gel was dried and exposed on X-Omat film (Eastman Kodak Co., Rochester, NY).

Immunoprecipitation of Cell Surface Integrins from BAEC Lysates

Lyophilized HSPG-core affinity purified proteins were reconstituted with PBS containing 1 mM PMSF and immunoprecipitated with rabbit polyclonal antibodies (anti-β1, anti-β3) and rabbit IgG. 100 μl of preabsorbed lysates was incubated with 50 μl of rabbit polyclonal antibody diluted 1:20 in 4% BSA in PBS for 1 h on ice, then incubated with 20 μl of cyanogen bromide–activated Sepharose CL-6B beads coupled to protein A for 1 h on ice. Beads were washed six times with washing buffer containing 0.5% sodium deoxycholate (Sigma Chemical Co.), 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4. Samples were boiled for 5 min in SDS sample buffer and subjected to SDS-polyacrylamide electrophoresis (SDS-PAGE; Laemmli, 1970) on gels prepared as a linear 3.5–12% gradient and under nonreducing conditions. Gels were dried and exposed on X-Omat x-ray film (Eastman Kodak Co.) at −70°C.

Immunoblotting

Samples separated by SDS-PAGE were transferred by cross-electrophoresis to a nitrocellulose membrane (Towbin et al., 1979), blocked with 4% BSA in PBS, and incubated with or without biotinylated-HSPG-core (sulfosuccinimidyl-6-(biotinamido) hexanoate, Pierce Chemical Co.) overnight at 4°C. After washing, the membrane was incubated with peroxidase-conjugated streptavidin (Pierce Chemical Co.) and color developed by DAB containing 0.01% hydrogen peroxide. Freshly prepared BAEC lysates were electrophoresed on SDS-PAGE and immunoblotted with anti β-1 and β-3 polyclonal antibody (described in detail in Basson et al., 1990). For further analysis of perlecan, blocked nitrocellulose membranes with transferred proteins were incubated with anti-laminin and fibronectin (10 μg/ml) for several hours. The membranes were washed, incubated with radioiodinated protein A (~10^6 cpm/ml) for an additional hour, washed, dried, and prepared for autoradiography.

Inhibition of Cell Attachment with Glycosaminoglycans, Antibody, and Synthetic Peptides

Soluble peptides (10–100 μg/ml) were directly added to the medium without fetal calf serum. Cycloheximide (2.5 μg/ml) was added 90 min before plating the cells. After incubating 120 min, cells were washed three times with PBS and reincubated with acid phosphatase substrate for an additional 1 h. The effects of heparin, de-*N*-sulfated heparin, dextran sulfate, chondroitin sulfates A, B, and C, and hyaluronic acid on BAEC attachment to HSPG-core, laminin, and fibronectin were evaluated at either a fixed concentration (1 μg/ml) to compare bound substrates or as a function of concentration (0–100 μg/ml) on core protein. Glycosaminoglycans (GAGs) were directly added to the medium containing cells and cell attachment was measured after 2 h.

Immunofluorescence Light Microscopy

Glass slides were coated with perlecan core and blocked with 4% BSA in PBS. Endothelial cells were cultured on these coated slides for up to 20 h. For incubations up to 5 h cells were treated with cycloheximide as previously described. For overnight incubations, cycloheximide was not used because of developing cytotoxicity. After incubations of substrate, cells were washed twice with cold PBS and then fixed and permeabilized with methanol for 4 min at −20°C, followed by acetone for 2 min at −20°C. The slides were covered with 5% normal goat serum in PBS for 15 min and then incubated with rabbit anti β1 (1:20), anti-β3 (1:20), anti-α-actinin (1:200), or rabbit nonimmune serum (1:20) for 2 h at room temperature. After washing with PBS containing 1 mg/ml BSA, 0.05% Tween 20, the slides were incubated with FITC-goat anti-rabbit IgG (1:100; Sigma Chemical Co.) for 1 h. The slides were again washed, and then mounted with coverslips using FITC-Guard (Testog, Chicago, IL). Slides were examined by epifluorescence microscopy.

Immuno-electron Microscopy

The procedure for immunocytochemistry localization using the unlabeled antibody peroxidase-anti-peroxidase technique was as described previously (Hayashi et al., 1987). Aorta from a C57B6 mouse was dissected and fixed in 4% formaldehyde (methanol free, EM grade; Polysciences, Inc., Warrington, PA) in 0.1 M phosphate buffer, pH 7.4, for 4 h. After fixation tissues were cut very thin and washed in 0.1 M phosphate buffer containing 7 M sucrose overnight at 4°C. The sliced tissue sections were treated with 1% hydrogen peroxide in 50% methanol for 30 min, washed, and covered with 5% BSA in 10 mM sodium phosphate, pH 7.4, containing 127 mM NaCl (PBS) for 15 min. The tissues were reacted with primary monoclonal antibody overnight at 4°C, then with goat-anti rat IgG (no cross-reaction to mouse IgG; Cooper Biomedical, Inc., Malvern, PA) at 1:20 dilution for 30 min and with rat peroxidase-anti-peroxidase (Jackson Immuno Research Laboratories, Inc., West Grove, PA) diluted 1:50 for 30 min at room temperature. After washing, tissues were fixed in 2% glutaraldehyde in PBS for 20 min at 4°C and washed in PBS. Sections were then placed in the DAB mixture containing 1% dimethyl sulfoxide but without hydrogen peroxide for 30 min and for an additional 3 min in the same mixture in the presence of 0.01% hydrogen peroxide. Sections were washed and reacted with 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 1 h to produce osmium black. Sections were dehydrated through a graded series of alcohols, embedded in Epon-Araldite, and polymerized at 60°C. Thin sections were cut with a diamond knife on a Sorvall Porter-Blum MT-2 ultra-microtome (Newtown, CT) and photographed with a Philips 420 electron microscope (Eindhoven, Holland). Negative controls for immunocytochemical staining included replacing the primary antibody with normal rat IgG in place of the primary antibody and processed in the same manner as above.

Results

Endothelial Cell Adhesion to Proteoglycan Core

Comparative attachment studies for perlecan, perlecan core protein, fibronectin, type IV collagen, and laminin immobilized on plastic wells were carried out with these components at a similar bound density. The attachment of endothelial cells on bacteriologic dishes coated with plasma fibronectin, type IV collagen, laminin, intact perlecan (HSPG) and HSPG-core were measured as a function of time (Fig. 1). Over a period of 3 h, cells were found to increasingly bind to protein substrate. The highest rate of binding occurred on fibronectin and type IV collagen. The average increase in

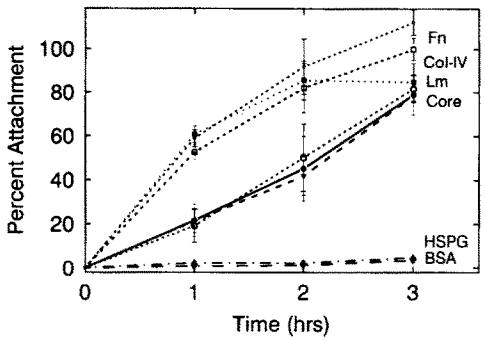

Figure 1. Time course of endothelial cell attachment to extracellular matrix components. Confluent endothelial cells, treated with cycloheximide (unless otherwise indicated), were plated, in the absence of serum, on bacteriological 96-well dishes previously coated with perlecan (*HSPG, open triangles, dash-dotted line,* $n = 3$), perlecan core (*Core, solid circles, solid line*; $n = 12$), core in the absence of cycloheximide (*open circles, short dashed line*; $n = 12$), laminin (*Lm, inverted solid triangles, solid line*; $n = 3$), fibronectin (*Fn, open squares, short dashed line*; $n = 3$), fibronectin without cycloheximide (*small open squares, short dashed line*; $n = 3$), collagen type IV (*Col-IV, solid squares, dotted line*; $n = 3$), and albumin (*BSA, solid triangles, dash-dotted line*; $n = 3$). Cells were incubated for the indicated times at 37°C in DME without fetal calf serum. After the incubation dishes were washed with PBS. Acid phosphatase activity in each well was measured by optical density which converted to the percentage of the cells attached to the matrix using standard curve of the cell number. Bars indicate standard deviations.

adhesion to perlecan core was nearly linear up to 3 h and furthermore was similar to that found with laminin. Adhesion to core or other components (cycloheximide-free plots shown only for core and fibronectin) was not dependent on protein synthesis because the adhesion levels were nearly the same in the absence and presence of cycloheximide (latter producing almost complete inhibition of protein synthesis). Little binding was observed on intact proteoglycan or albumin. The possibility that the heparitinase used to prepare isolated core, or a contaminant in the enzyme preparation, contributed to cell adhesion was evaluated by plating cells on immobilized heparitinase (0.03 cu/ml coat, the same concentration present in the core preparation): cell adhesion was found to be essentially identical (1.6, 2.3, and 3.7% at 1, 2, and 3 h) to the albumin control. The basis for the poor adhesion to intact perlecan was evaluated as described in Figs. 11 and 12. The effect of cell attachment on cell morphology was examined (data not shown). Cells which attached to fibronectin, laminin, and core were found to develop a "spread" (flattened fusiform) morphology, interpreted as a cytoskeletal rearrangement in response to attachment. Those few cells which bound intact perlecan retained a rounded morphology.

Characterization of the Core Substrate and Inhibition of Cell Adhesion with Core-specific Antibody

By SDS-PAGE (Fig. 2), the core protein migrated as a single major species with a molecular weight of ~450 kD as previously observed (Yurchenco et al., 1987). No species the size of laminin (~400, 200 kD), entactin (150 kD), type IV collagen (185, 170 kD), fibronectin (220 kD) or vitronectin was observed. Affinity-purified core antibody reacted with core, but not laminin, entactin, fibronectin, vitronectin, type IV collagen, or type I collagen by immunoblot and/or competition ELISA assay (Fig. 2). Laminin A chain and fibronectin-specific antibodies (Fig. 2) did not cross-react with protein in the core preparation. The ability of core-specific antibody to block cell adhesion to core was then evaluated (Fig. 3). This antibody blocked most (>75% at the highest concentration shown and under presaturating conditions) cell adhesion to core protein in a concentration-dependent manner, but had little or no effect on laminin, fibronectin, vitronectin, or type IV collagen substrates.

Inhibition of Cell Adhesion with Integrin Antibodies

mAb (AIIB2) to β1 integrins partially but substantially inhibited cell attachment (Fig. 4 *A*). mAb (LM609) to the vitronectin receptor (αvβ3 integrin) produced no inhibition of cell adhesion when used alone. However, a mixture of these two antibodies produced almost total inhibition of cell adhesion. Furthermore, maximal inhibition with the β1-specific reagent permitted the αvβ3-specific reagent to block the remaining cells from adhering to the perlecan core. EDTA, which can inhibit the binding of many integrins, almost completely blocked endothelial cells adhesion (Fig. 4 *B*). The blocking integrin antibodies were obtained as ascites. We purified an aliquot of AIIB2 to near homogeneity by ammonium sulfate precipitation and found it produced the same degree of inhibition when antibody concentrations in the pure preparation and ascites were matched (data not shown). Thus, in the concentration range studied, ascites did not appear to have an adverse effect on cell adhesion compared to pure reagent. The high concentration of purified antibody obtained permitted us to evaluate maximal inhibition of adhesion to core and, at both 75 and 150 μg/ml antibody, 17% of cells adhered to perlecan core (plot not shown). We concluded that endothelial cell adhesion to perlecan is mediated by integrins, and furthermore that the principal integrins involved possess β1 and β3 chains. The latter receptor, because of the antibody specificity (Cheresh and Spiro, 1987), is likely to be the vitronectin receptor, αvβ3.

Inhibition of Cell Adhesion to Proteoglycan Core with Synthetic Peptides

Cell adhesion to the HSPG-core was significantly inhibited (one half to two thirds) by synthetic peptides containing arg-gly-asp (RGD) but not by a control peptide (GRGESP) (Fig. 5 *A*). The core-specific peptide (c-RGD) was also effective as an inhibitor, exerting its effect both free or covalently coupled to albumin. A similar effect was observed with tissue culture plates coated with fibronectin (Fig. 5 *B*). Endothelial cells were also found to bind directly to a synthetic peptide containing 13 amino acids from the RGD region of perlecan and coupled to an albumin support via an NH_2-terminal cysteine (c-RGD-BSA, Fig. 5 *C*). This latter adhesion, as expected, was almost completely inhibited by soluble c-RGD. We concluded that endothelial cells bind to at least two topographical sites in the core protein. One of these sites is the single RGD triplet present in the lamininlike region. The binding at this site was independent of residues flanking the RGD triplet (compare sequences of, and inhibition of cell attachment by, the fibronectin and core-specific RGD pep-

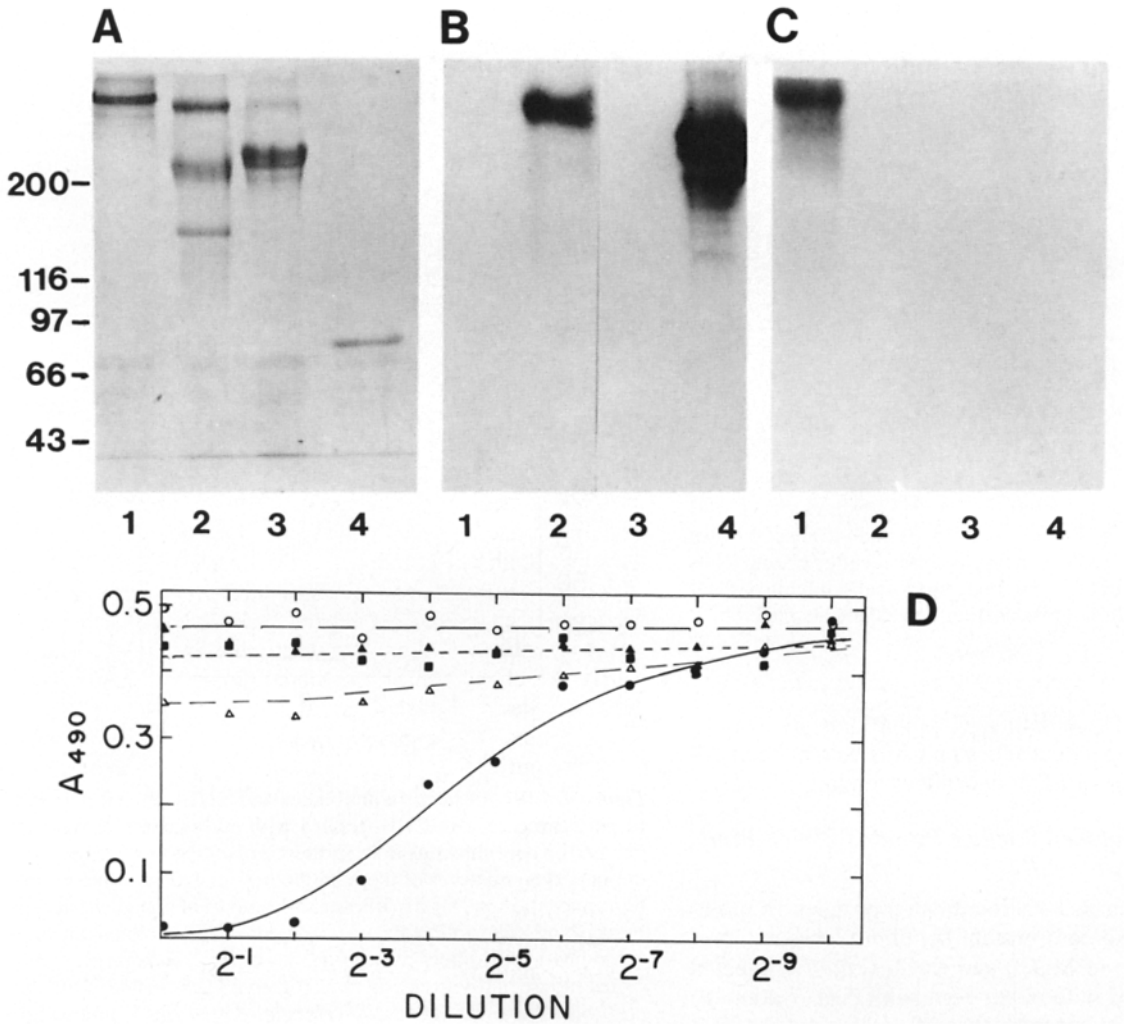

Figure 2. Characterization of perlecan core (*A* and *B*) and core-specific antibody (*C* and *D*). (*A*) SDS-PAGE Coomassie blue stained gels of reduced mouse perlecan core (lane *1*), mouse laminin/entactin (lane *2*, with bands at ~400, 200, and 150 kD), rat fibronectin (lane *3*) and mouse vitronectin (lane *4*). (*B*) Autoradiograms (Western blot) of electrophoretically transferred proteins incubated with rabbit anti-laminin A chain (lanes *1* and *2*) and rabbit anti-fibronectin (lanes *3* and *4*). One μg of each protein was separated under reducing conditions by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose, after blocking with BSA, was incubated with 10 μg/ml affinity-purified antibody and labeled with ^{125}I-protein A. Core protein preparation analyzed in lanes 1 and 3; laminin control, lane 2; fibronectin control, lane 4. (*C*) Core-specific antibody immunoblots (Western). 1 μg of perlecan core (lane *1*), laminin/entactin (lane *2*), rat fibronectin (lane *3*), and mouse vitronectin (lane *4*) were separated under reducing conditions by SDS-PAGE, transferred to nitrocellulose, blocked, incubated with 10 μg/ml affinity-purified rabbit anti-perlecan core, and detected with ^{125}I-protein A. Reaction is observed only with core protein. (*D*) Competition ELISA assay to further evaluate specificity of core-specific antibody. 96-well plates were coated with 0.1 μg/well of perlecan and incubated with anti-core antibody (4 μg/ml) in the presence of serial twofold dilutions of soluble ligands (undiluted = 300 μg/ml). (*Ligands*) Perlecan (*closed circles*), laminin (*closed squares*), entactin (*open circles*), type IV collagen (*open triangles*), type I collagen (*closed triangles*).

tides). The other site(s), detected on core in the presence of inhibiting concentrations of soluble RGD peptides, are RGD-insensitive.

To determine the relative contributions of the two integrins to RGD-dependent and RGD-independent adhesion, we evaluated the ability of the two integrin blocking antibodies for cells plated onto immobilized synthetic peptide (Fig. 6 *A*) or onto the core protein of perlecan in the presence of maximally inhibiting concentrations of RGD peptide (Fig. 6 *B*). On the cRGD-BSA substrate, anti-β1 antibody produced some inhibition of adhesion whereas anti-$\alpha v\beta 3$ produced no inhibition alone. On the other hand, a mixture of the two antibodies inhibited most cell adhesion and, thus, the $\alpha v\beta 3$ antibody appeared to play a significant role in RGD-mediated adhesion. RGD peptide (GRGDSP) produced a ~50% baseline inhibition of cell adhesion to core (Fig. 6 *B*). Under these conditions, anti-β1 antibody produced essentially complete inhibition of cell adhesion at concentrations (5 μg/ml total protein) which produced only partial inhibition on core substrate alone or on immobilized RGD peptide (compare with Figs. 4 *A* and 6 *B*). Furthermore, the addition of anti-$\alpha v\beta 3$ antibody produced little or no additional enhancement

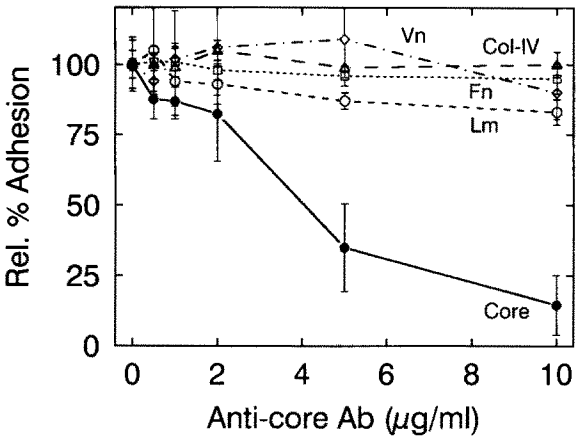

Figure 3. Inhibition of endothelial cell adhesion with core-specific antibody. Wells were coated with perlecan core (*solid circles*; n = 6), laminin (*open circles*; n = 3), fibronectin (*open diamond*; n = 3), type IV collagen (*open triangles*; n = 3) and vitronectin (*open diamonds*; n = 3). Cell adhesion was assayed 2 h after plating (cells treated with cycloheximide). Antibody specifically inhibited adhesion to core protein in a concentration-dependent manner.

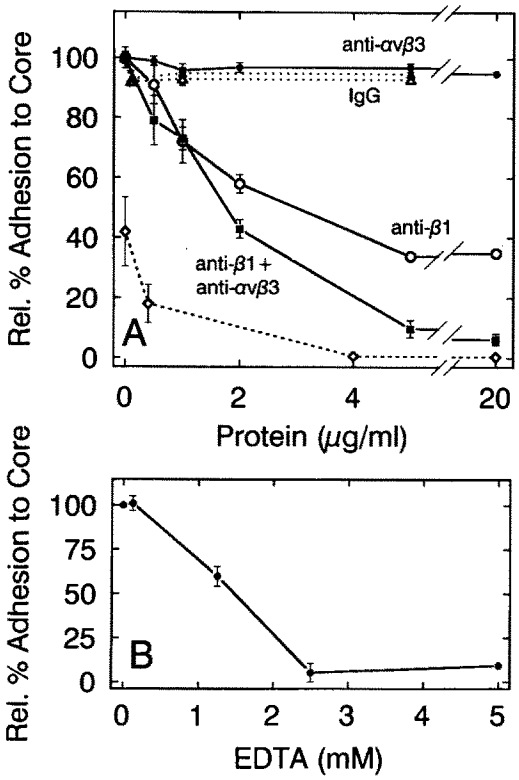

Figure 4. Effect of integrin antibodies and EDTA on cell adhesion to perlecan core. (*A*) Cells (treated with cycloheximide) were incubated on core substrate in the presence of antibodies to two different integrins, mixtures of these antibodies, or IgG at different protein concentrations for 2 h followed by removal of nonadherent cells by washing. The following were evaluated: monoclonal rat anti-ectodomain of β1 integrins (*anti-β1; open circle, solid line*), monoclonal mouse anti-vitronectin receptor, αvβ3 (*anti-αvβ3; solid circle, solid line*), rat IgG (*solid triangle, dotted line*), mouse IgG (*open triangle, dotted line*), anti-β1 mixed with anti-αvβ3 in a constant 1:1 ratio (*solid square, solid line*), and anti-β1 maintained at a constant 100 μg/ml whereas anti-αvβ3 was varied (*open diamonds, short dashed line*). Anti-β1 antibody blocked ~60% of cell adhesion as ascites. Purified antibody saturated at 75 μg/ml with 83% inhibition. A mixture of antibodies produced complete inhibition (*open diamonds*) whereas anti-αvβ3 produced no inhibition on its own. (*B*) Inhibition of cell adhesion with the chelating agent, EDTA. Chelation of divalent cation produced almost complete inhibition of adhesion.

of this inhibition. These data suggest that only β1 integrin mediates RGD-independent adhesion while both β-1 and β-3 integrins play a role in RGD-dependent binding.

Characterization of Cell Surface Proteins Which Bind HSPG Core Protein

A fraction of cell surface-radioiodinated proteins from endothelial cells bound core protein by affinity chromatography in the presence of $MgCl_2$ and $CaCl_2$ (Fig. 7). Labeled proteins were eluted with either high salt (Figs. 7 *a* and 8) or EDTA (Fig. 8). In contrast, the cell lysates did not bind to an albumin control column (Fig. 7 *b*). SDS-PAGE analysis (Fig. 8) of iodinated endothelial cells lysates eluted from HSPG-core affinity chromatography revealed bands with molecular masses of 147, 116, 85, and also 60- and 35-kD bands. EDTA was more selective for the larger species (85–147 kD; Fig. 8). Immunoprecipitation of these HSPG-core purified proteins from total cell lysate and from core-affinity purified lysate with integrin antibodies demonstrated a shared larger species (147 kD), the alpha chain subunit, associated with the 116-kD β1 chain and with the 85-kD β3 chain (Figs. 8, and 9). The ability of the protein eluted from HSPG-core column to directly bind the core protein was evaluated by immunoblotting (Fig. 10). The core was found to react directly with the 116-kD β1 cell surface protein. The lack of binding to the 85- and 147-kD bands may be explained by a lower affinity of interaction or a loss of the conformation required for binding during SDS-PAGE.

Inhibition of Cell Attachment with Glycosaminoglycans

As described (Fig. 1), endothelial cells adhered to the core protein of perlecan, but that this adhesion was greatly reduced if the heparan sulfate chains were not first removed with heparitinase. Because perlecan can possess heparan sulfate chains of variable length, variable degrees of sulfation, or even completely free of heparan sulfate, we considered the hypothesis that these chains serve a post-translational regulatory function for adhesion. We addressed the possibility that cell adhesion is inhibited by heparin/heparan sulfate chains regardless of whether they are covalently linked to core, that this inhibiting activity is glycosaminoglycan-specific, and that the inhibition mechanism may not be confined to perlecan alone (Fig. 11).

Cell adhesion to core protein was therefore evaluated in the presence (Fig. 11 *A*) of heparin, chondroitin sulfates A, B, C, bovine kidney heparan sulfate, EHS heparan sulfate, and dextran sulfate. Heparin, EHS heparan sulfate, kidney heparan sulfate, and dextran sulfate, unlike the other components tested, inhibited cell attachment to the HSPG-core. Heparin was the most effective agent and only the more highly sulfated polysaccharides had activity. We compared

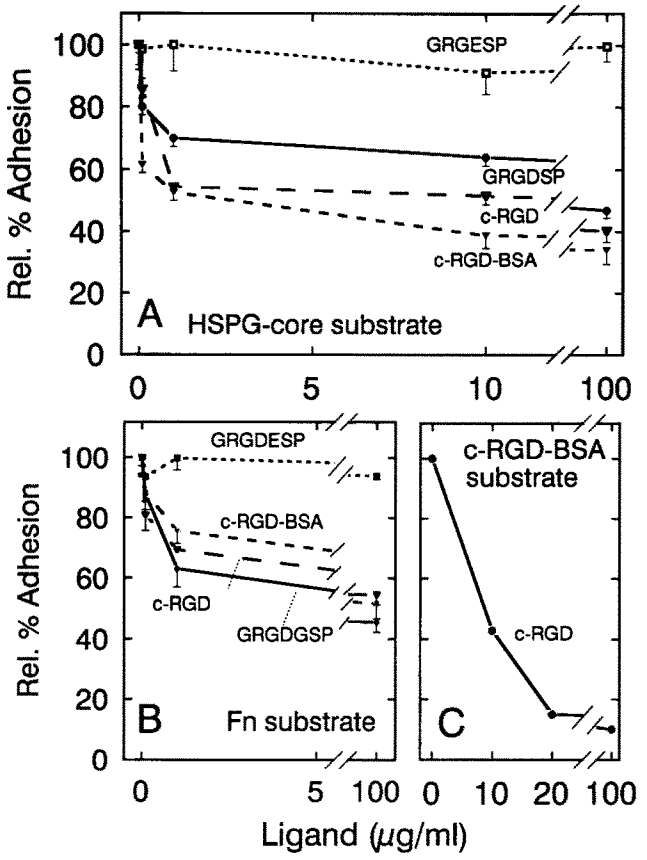

Figure 5. Effects of synthetic peptides on cell adhesion. (*A*) Inhibition of cell adhesion to perlecan core. Soluble short synthetic peptides GRGDSP, GRGESP, synthetic peptide containing a 13-amino acid sequence from the RGD region in perlecan (*c-RGD*: *upside down triangle, long dashed line*), and c-RGD-BSA covalently bound complex were added directly to the medium. Cell attachment was measured after 120 min. RGD specifically, but incompletely, inhibited cell attachment to both HSPG-core and fibronectin. Addition of core-RGD peptide to BSA-core-RGD coated dishes similarly inhibited cell attachment in a dose-dependent manner. While inhibition was observed with GRGDSP (*solid circle, solid line*), it was not observed with GRGESP (*open square, short dashed line*). (*B*) Inhibition of cell adhesion to fibronectin with GRGDSP, c-RGD, and c-RGD-BSA but not GRGDESP. RGD-containing peptides, regardless of flanking sequences, partially inhibit cell adhesion to both perlecan core and fibronectin. (*C*) Endothelial cells bound directly to c-RGD-BSA immobilized on plastic. This adhesion was almost completely inhibited with c-RGD peptide. Cells treated with cycloheximide for all three panels.

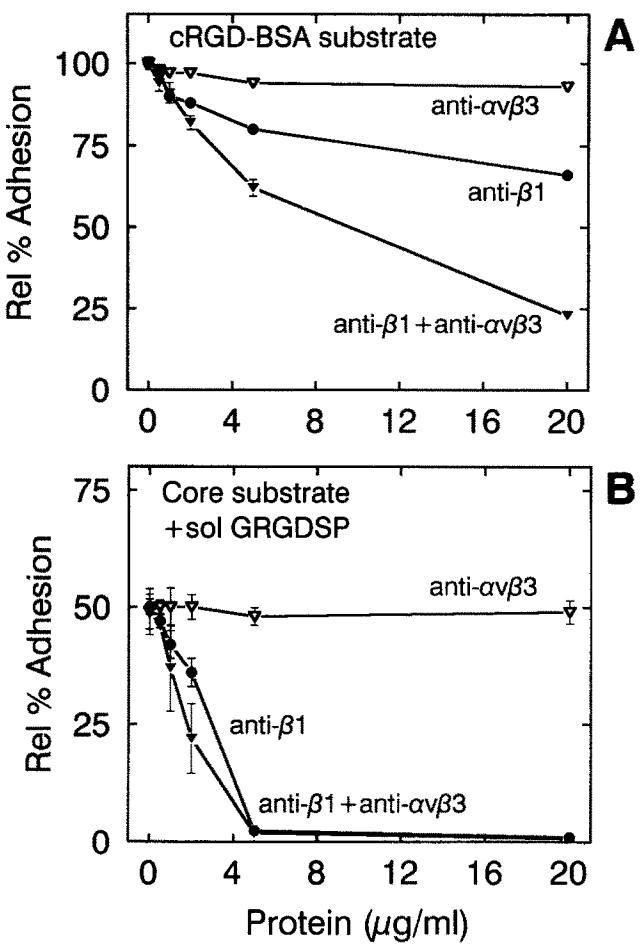

Figure 6. Evaluation of cell adhesion to RGD-substrate (*A*) and to perlecan core in the presence of 100 μg/ml RGD peptide (*B*). (*A*) Anti-β1 antibody (*solid circles*) partially inhibited cell adhesion to c-RGD-BSA whereas anti-αvβ3 (*open upside down triangles*) did not. A mixture of the two antibodies (*solid upside down triangles*) produced substantial inhibition. (*B*) RGD-dependent binding to core was maximally inhibited with RGD peptide (100 μg/ml). Anti-β1 antibody almost completely inhibited cell adhesion under these conditions and at substantially lower concentration compared to core in the absence of RGD. Furthermore, the addition of anti-αvβ3 produced little or no enhancement of inhibition, unlike that observed with c-RGD-BSA substrate.

endothelial cells attachment to HSPG-core, laminin, and fibronectin (Fig. 11 *B*). Similar inhibitions were observed for all three components at low heparin concentrations (1 μg/ml). This inhibition was lost if de-*N*-sulfated heparin, which lacks N-substituted but retains the O-substituted sulfates, was used instead of heparin. As described above, immobilized perlecan synthetic peptide c-RGD supported cell adhesion and soluble RGD peptides partially blocked cell attachment to perlecan core. We compared the ability of heparin to inhibit endothelial cells adhesion to perlecan core in the presence and absence of RGD peptide and to immobilized c-RGD-BSA (Fig. 11 *C*). While heparin inhibited cell adhesion to core, it did not inhibit cell adhesion to c-RGD peptide, even at high concentration. Furthermore, heparin blocked residual cell adhesion to core in the presence of maximally inhibiting concentrations of soluble RGD peptide. We concluded that endothelial cells needed to recognize a non-RGD domain in perlecan for heparin to be capable of inhibiting adhesion.

We questioned whether there was a relationship between the binding of heparin to substrate and heparin inhibition of cell adhesion. Relative heparin binding (Fig. 12) to laminin E3 (main heparin binding domain; Yurchenco et al., 1990), BSA, perlecan core protein, and fibronectin were measured by incubating nitrocellulose blots of these proteins with radiolabeled heparin. As expected, binding to laminin E3 and fibronectin was observed. The core RGD-peptide conjugated to albumin also bound heparin (this latter binding may be ex-

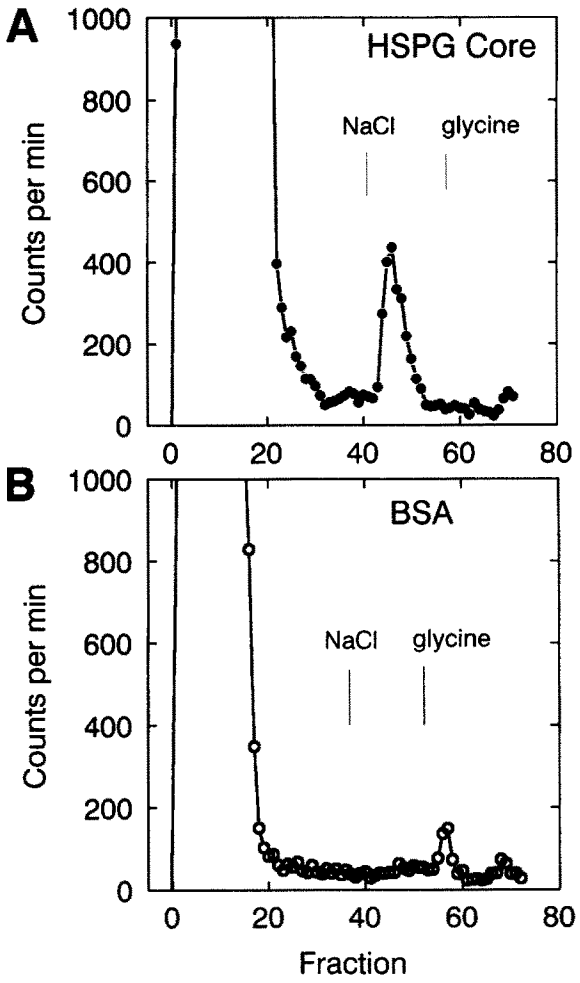

Figure 7. Identification of ^{125}I-surface-labeled bovine endothelial cells (endothelial cells) proteins by HSPG-core affinity chromatography. Endothelial cells cell surface membranes, surface radiolabeled and extracted by octylglucoside, were chromatographed on HSPG-core (*A*) and BSA (*B*) coupled Sepharose 4B affinity columns. Proteins were eluted with a linear 0.5–1 M NaCl gradient in the presence of $MgCl_2$ and $CaCl_2$ or EDTA followed by 0.1 M glycine and 6 M urea.

plained by the presence of two basic residues in each RGD 14mer with many peptides bound to each albumin molecule, producing local areas of increased basic charge density). On the other hand, no binding was detected to perlecan core protein or to (unconjugated) albumin. We concluded it unlikely there is any relationship between the ability of protein substrate to bind heparin and the ability of heparin to inhibit cell adhesion to substrate. Fibronectin, laminin, and RGD peptide conjugated to BSA each bind heparin, but cell adhesion is inhibited only for the first two. Perlecan core does not bind heparin (noncovalently), yet it is quite sensitive to heparin/heparan sulfate inhibition of cell adhesion.

Localization of α-Actinin, β1, and β3 Integrins in Endothelial Cells Adherent to Perlecan Core Substrate

Endothelial cells were allowed to adhere to and spread on glass slides coated with perlecan core and blocked with BSA. After fixation and antibody staining, the cells were examined by immunofluorescence microscopy (Fig. 13). By 1 h incubation, β1 integrin were observed in punctate and elongated structures at the cell periphery with more diffuse staining in the perinuclear region. A lesser degree of such focal staining was observed for α-actinin and β3 integrin with most of the staining more diffuse. By several hours, increasing amounts of all three proteins were observed in these discrete peripheral structures, suggestive of focal contacts. The focal structures were observed to be present the following day. These data suggest that β1 integrins, and β3 integrins more slowly, accumulate in focal contacts or focal contact-like structures in response to cell adhesion and spreading on perlecan core protein.

Localization of Perlecan in the Basement Membrane of Mouse Aorta

Ultrastructural immunoperoxidase stain showed the flat aortic endothelial cells adherent to the underlying basement membrane. Perlecan core antigen was localized in the lamina densa of this basement membrane. Other than the presence of some intracellular label in endothelial vesicles (presumably in secretory vesicles), antibody reaction product was not detected elsewhere. Nonimmune antibody gave almost no detectable staining (Fig. 14).

Discussion

Perlecan, a high molecular weight heparan sulfate proteoglycan, has been identified in a wide variety of basement membranes (Kato et al., 1987) and isolated from sources such as mouse EHS tumor (Paulsson et al., 1987*a*) and endothelial cell culture medium (Saku and Furthmayr, 1989). By EM the core protein has the appearance of a tandem array of five or six globular regions separated by thinner necklike links (Paulsson et al., 1987; Yurchenco et al., 1987). Recently, the complete sequence for mouse perlecan (Noonan et al., 1991) was deduced from analysis of overlapping cDNA clones. On the basis of this sequence, perlecan has been divided into five domains: a heparan sulfate region (domain I) followed by an LDL-receptorlike region (domain II), a laminin short-arm region with three globules and three intervening EGF-like repeats (domain III), an N-CAM-like region (domain IV) and finally laminin A-chain G domainlike regions (domain V) near the COOH terminus. Analogous to other multidomain extracellular matrix molecules (e.g., fibronectin), this type of structure suggests the existence of a variety of domain-specific structural and cell-interactive functions. While nonintegrin cell surface proteins (38 and 80 kD) from hepatocytes have previously been reported to bind to the core protein of the basement membrane HSPG (Clement et al., 1989, Clement and Yamada, 1990), the presence of an RGD sequence (amino acid residues 998–1,000) in the second globule of the lamininlike domain III (Noonan et al., 1988, 1991) suggested that endothelial cell adhesion to HSPG could be mediated by the integrin family.

In this study, we evaluated the adhesive properties of perlecan for aortic endothelial cells, cultured primary explanted cells which have been well-characterized with respect to their interactions with other extracellular matrix components. These matrix macromolecules have been found to

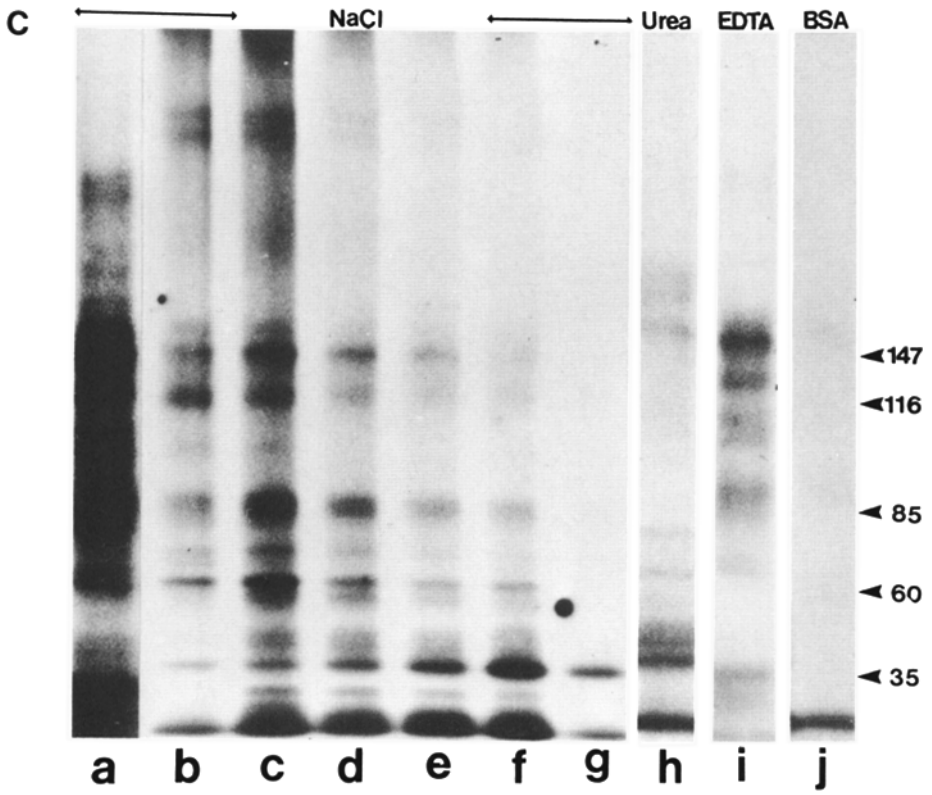

Figure 8. Autoradiogram of labeled cell surface protein eluted from HSPG-core affinity column (lanes *a–i*) and BSA column (lane *j*) analyzed by SDS-PAGE (3.5–12% linear polyacrylamide gradient). Lanes: (*a*) unbound fraction, (*b*) bound fractions 37–39, (*c*) 40–42, (*d*) 43–45, (*e*) 46–49, (*f*) 50–55, (*g*) 56–65, (*h*) peak fraction subsequently eluted with 6 M urea; (*i*) peak fraction eluted with 5 mM EDTA in separate run; (*j*) peak fraction eluted with NaCl from control BSA column.

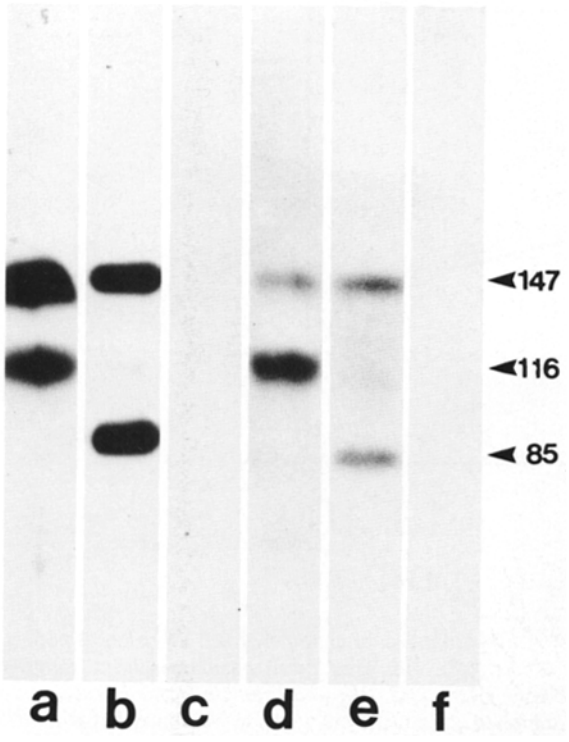

Figure 9. Immunoprecipitation of integrin from endothelial cell lysates (lanes *a–c*), and HSPG-core purified endothelial cell lysates (lanes *d–f*). ^{125}I-labeled endothelial cell lysates from confluent endothelial cells were purified with HSPG-core affinity chromatography. The peak fractions eluted with NaCl gradient were immunoprecipitated with rabbit polyclonal anti-β1, (*a* and *d*), anti-β3 (*b* and *e*), and rabbit IgG (*c* and *f*), followed by incubation with protein A–Sepharose. The proteins released from the beads with SDS were subjected to PAGE (linear 3.5–12% gradient) under nonreducing conditions β1 (147, 116 kD) and β3 (147, 85 kD) integrins were immunoprecipitated from endothelial cells cell lysates which bound HSPG-core.

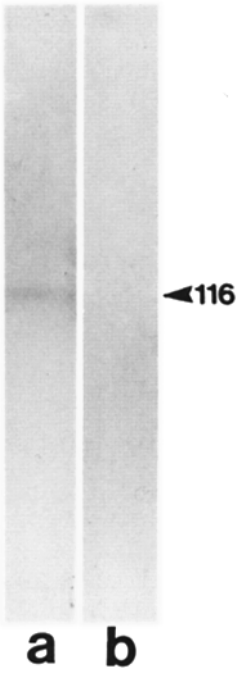

Figure 10. Binding of HSPG-core purified endothelial cell surface protein to HSPG-core. The peak fraction of endothelial cells cell lysates were eluted from a HSPG-core column with a NaCl gradient, pooled and subjected to SDS-PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane and incubated with (lane *a*) or without (lane *b*) biotinylated-HSPG-core overnight at 4°C. After washing the membrane was incubated with peroxidase-conjugated streptavidin and colored by DAB containing 0.01% hydrogen peroxide. Core reacted specifically with the 116-kD cell surface protein.

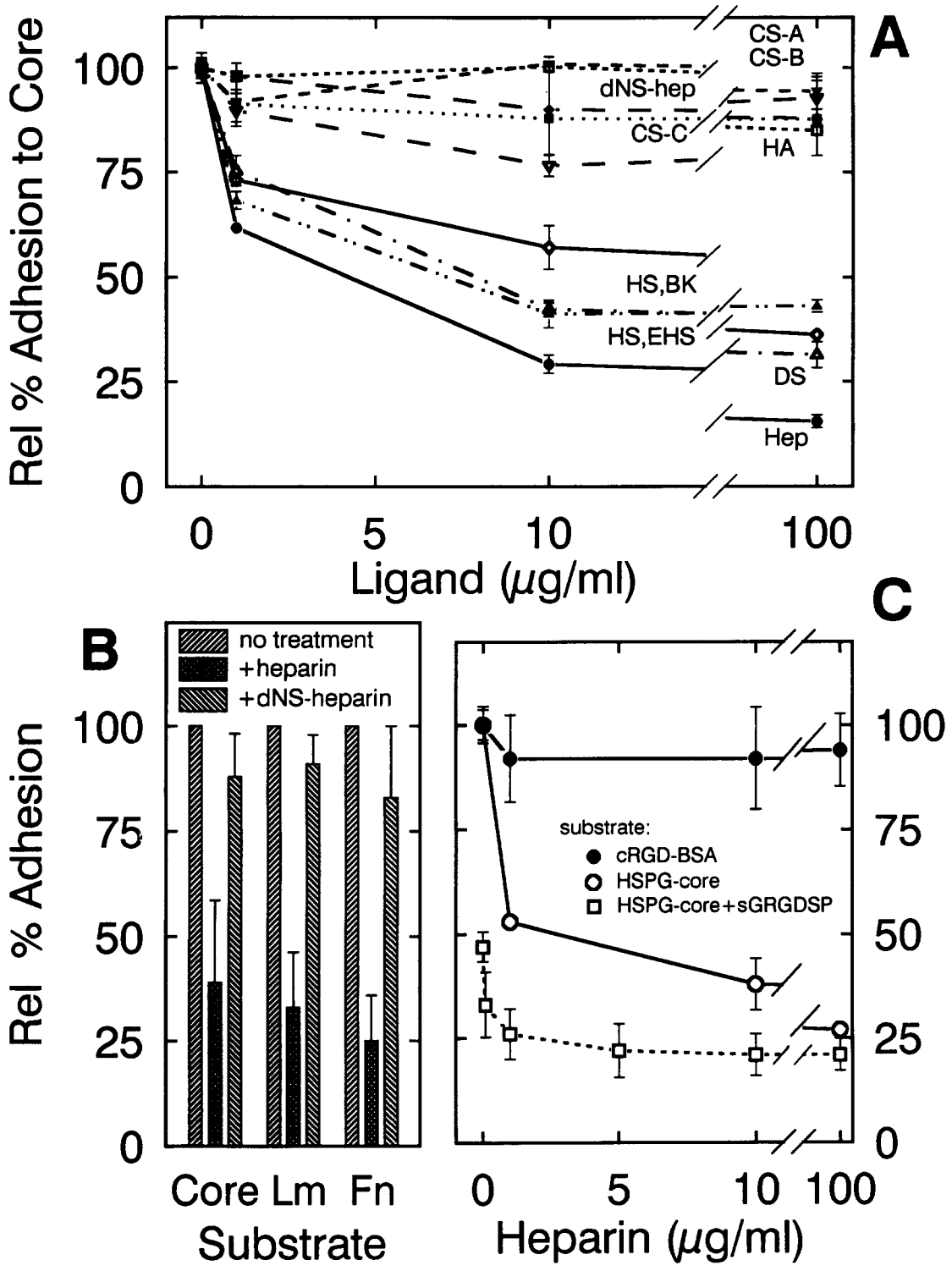

Figure 11. Effects of exogenous glycosaminoglycans on endothelial cell adhesion. (*A*) Inhibition of endothelial cell adhesion to perlecan core was determined for different glycosaminoglycans after 2 h. Heparin (*hep: solid circles, solid line*); heparan sulfate isolated from EHS perlecan (*HS, EHS; open diamonds, solid line*), heparan sulfate isolated from bovine kidney (*HS, BK: solid triangle, dashed double dotted line*), and dextran sulfate (*DS: open triangle, dashed dotted line*) substantially inhibited cell attachment while de-*N*-sulfated heparin (*dNS-hep: solid diamond, long dashed line*), chondroitin sulfates *A, B, C* (*CS-A, CS-B, CS-C: upside-down closed triangle, medium dashed line; side down open triangle, short dashed line; solid square, dotted line, respectively*), and hyaluronic acid (*HA: open square, short dashed line*) did not. Average and standard deviation of three measurements shown for each incubation. (*B*) Effects of heparin and de-*N*-sulfated heparin on endothelial cells. Attachment to perlecan core, laminin (Lm), and fibronectin (Fn). Heparin (Hep) and de-*N*-sulfated heparin (dNS-heparin) were added to the medium before cell attachment. Addition of 1 μg/ml produced significant inhibition of cell attachment to the core, laminin and fibronectin (at 2 h binding) whereas addition of the same amount of de-*N*-sulfated heparin had no significant effect. (*C*) Substrate requirement for heparin inhibition. Cell adhesion was evaluated on the following immobilized substrates in the absence

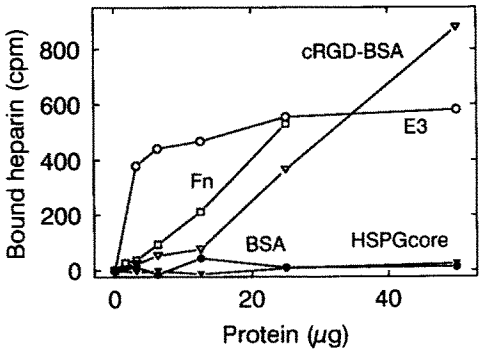

Figure 12. Heparin binding to cell substrates. Protein samples used as cell substrates were dot-blotted (0–50 μg/well) onto a 0.45-μm nitrocellulose sheet using a 96-well vacuum manifold. The sheets were then detached from the manifold, washed in PBS, incubated with ^{125}I-heparin (~15 kD; 1 mg/ml; 2.4 × 10^5 cpm/μg) for several hours at room temperature, washed with several changes of PBS, and air-dried. The sample dots were cut out and radioactivity was determined with a gamma counter. Background counts (162), determined from wells without protein, were subtracted. Heparin binding to cell substrates. The substrates were the heparin-binding laminin fragment E3 (*open circles*), fibronectin (*open squares*), perlecan core (*solid upside down triangles*), BSA (*solid circles*), and c-RGD peptide conjugated to albumin (*open upside down triangles*).

influence cell proliferation, cell migration, cell shape, and cytoskeletal organization (Palotie et al., 1983; Madri and Stenn, 1982; Madri and Pratt, 1986). Several integrins (β1 and β3 class) have been implicated in the mediation of endothelial adhesion to the basement membrane components laminin and type IV collagen (Basson et al., 1990). Two of the integrins involved in these interactions are α5β1 and αvβ3 (Basson et al., 1992).

We have found that the perlecan core protein supports both the attachment and spreading of cultured endothelial cells at a level comparable to that of laminin, but below that of fibronectin and type IV collagen, and confirmed that perlecan is a component of aortic endothelial basement membrane. There are several lines of evidence to support the hypothesis that this adhesion is directly mediated through the core protein. First, cell adhesion can be detected as early as 1 h, even when protein synthesis has been almost completely inhibited. Second, adhesion is selectively blocked by core-specific antibody. Third, β1 and β3 integrins bind directly to core protein by affinity chromatography and core protein binds directly to a cell surface protein identical in migration (116 kD) to β1 integrin.

The profound inhibition of cell adhesion with EDTA implicated the integrins as major receptors in the process of endothelial cells adhesion to perlecan (Hynes, 1987; Ruoslahti and Pierschbacher, 1987). This adhesion appeared to be a specific consequence of a cell-core interaction because antibody specific for core blocked adhesion to core, but not to other components. Well over half of this adhesion could be inhibited with a β1-specific antibody, implicating this integrin class as a major receptor. While an αvβ3 specific blocking antibody produced no inhibition on its own, both antibodies together essentially completely inhibited cell adhesion and, thus, β3 antibody considerably enhanced the efficacy of the first reagent. A possible thermodynamic mechanism for this effect is that the β1 integrin binds to the perlecan core with relatively higher affinity and, therefore, a selective block of these receptors results in a partial cell dissociation (because only low affinity β3 receptors remain to interact with substrate), while a selective block of low affinity β3 integrin leaves the high affinity β1 receptors firmly adherent. Immunostaining of endothelial cells with mAbs to the two integrins revealed that all cells, or nearly all cells, possessed both integrins, i.e., the two integrin classes were not sorted into two populations of cells. The identification of β1, and with time β3, integrin epitopes localized in what appear to be cellular focal contacts suggests that these integrins both play a role (β1 may be the first temporally) in the transition of adhesion to spreading.

Cell surface-labeled lysates which bound to the core protein were characterized and found to be enriched in β1 and β3 integrins. These integrins (~116 and ~85 kD, respectively), each in association with a larger α-chain (~147 kD), are the same as those identified in interactions with laminin (Basson et al., 1990). The α-chain is the same size as that immunoprecipitated with α5β1 specific antibody from aortic endothelial cells (Basson et al., 1992) and may be the same; however, further investigation is required to confirm this. Because β1 integrins are found mediating RGD-dependent and RGD-independent interactions, there may be two or more α-chains of the same, or nearly the same, molecular weight. The core protein selectively bound to the β1 band in a nitrocellulose blot and was evidence for a direct binding interaction between core and receptor. However, no interaction with the β3 chain could be detected under the conditions of the assay. This might be explained on the same basis as the greater inhibition of adhesion produced by the anti-β1 antibody, i.e., that the core binds to one integrin with higher affinity than the other; however, a conformational dependency for β3 receptor, in which binding activity is lost after separation of the two chains, cannot be excluded.

About half of cell adhesion could be inhibited with RGD peptides and integrin antibodies were found to inhibit cell adhesion to both immobilized RGD-peptide as well as to core protein whose RGD sites were blocked by soluble peptides. β1 integrin, in contrast to β3 integrin, appears to be selectively required for RGD-independent adhesion to perlecan because only the AIIB2 reagent had any inhibition activity for cell attachment to core when maximally blocked with soluble RGD peptide. While β1 integrin appears to play a role in both RGD-dependent and independent adhesion, we cannot determine whether it is the same integrin dimer which mediates both, or, whether two different β1 integrin dimers are involved each with a similar-sized alpha chain not resolved by SDS-PAGE (e.g., von der Mark et al., 1991). β1

or presence of inhibiting concentrations of GRGDSP peptide: core-RGD synthetic peptide (14-mer) covalently coupled to BSA (*c-RGD-BSA, solid circles*), perlecan core protein (*HSPG-core, open circles*), and perlecan core protein in the presence of 100 μg/ml soluble GRGDSP peptide (*open squares*), each in the presence of increasing concentrations of heparin. While cell adhesion to core was inhibited both in the absence and presence of RGD peptide, adhesion to immobilized synthetic peptide was not affected.

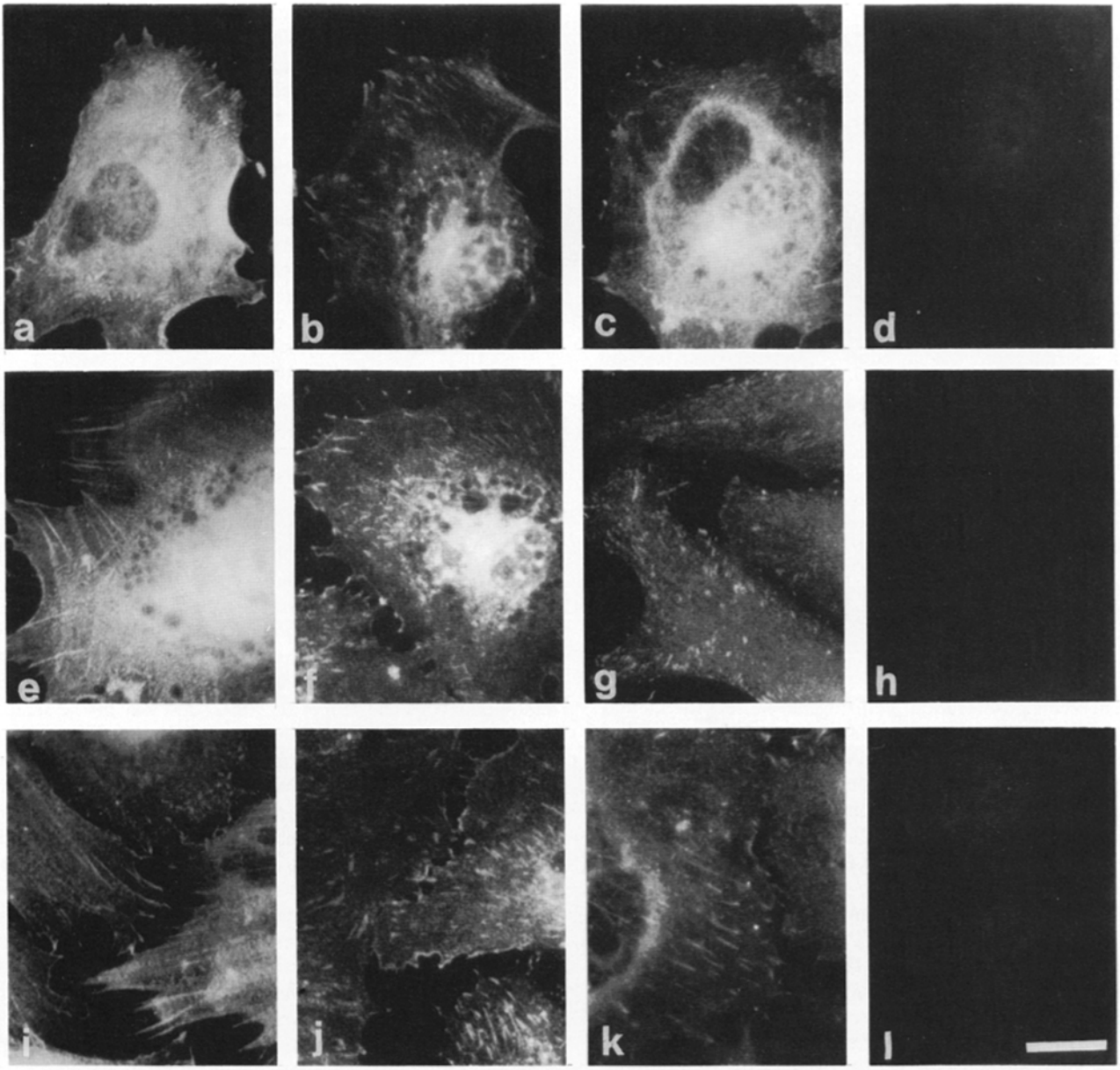

Figure 13. Immunolocalization of integrins on endothelial cells adherent to perlecan core protein. Aortic endothelial cells were cultured in the absence of serum on glass slides previously coated with core protein and blocked with BSA. Cells were evaluated after 1 h (*a–d*), 5 h (*e–h*) and 20 h (*i–l*) incubation. For 1 and 5 h, but not for 20 h, the cells were treated with cycloheximide. The adherent cells were then fixed and permeabilized, and stained with rabbit antiserum specific for α-actinin (*a*, *e*, *i*), β1 integrin (*b*, *f*, *j*), β3 integrin (*c*, *g*, *k*) as well as nonimmune serum (*d*, *h*, *l*). Focal punctate to linear staining structures, most prominently at the cell periphery, are detected as early as 1 h but increase in number with time for all three antibodies. Bar, 20 μm.

integrin-mediated RGD-independent adhesion has been demonstrated in several other biological systems including the α4β1/VCAM-1 lymphocyte–endothelial interaction (Elices et al., 1990), the α4β1/IIICS lymphocyte– and melanoma cell–fibronectin interactions (Guan and Hynes, 1990), and the α3β1, α4β1, α5β1, α6β1/cell-bacteria invasion interactions (Isberg and Leong, 1990).

A surprising finding was the sensitivity of cell binding to the presence of core-bound heparan sulfate or exogenous heparin and heparan sulfate. This inhibition was specific because it was not observed with other polyanionic polysaccharides such as hyaluronic acid and chondroitin sulfates and required the presence of *N*-sulfation. A prediction of this observation is that perlecan will not support endothelial cell adhesion unless the heparan sulfate chains have first been removed, are expressed with low sulfation, or are otherwise blocked (e.g., by binding to other basic proteins). While there is no direct evidence for the physiological significance of the endothelial–core interaction, there are indirect lines of evidence that support such a role. Human colon carcinoma cells have been shown to secrete the perlecan precursor protein (core free of heparan sulfate) into the extracellular space (Iozzo and Hassell, 1989), fibroblasts can secrete perlecan as a chondroitin sulfate proteoglycan (Hassell et

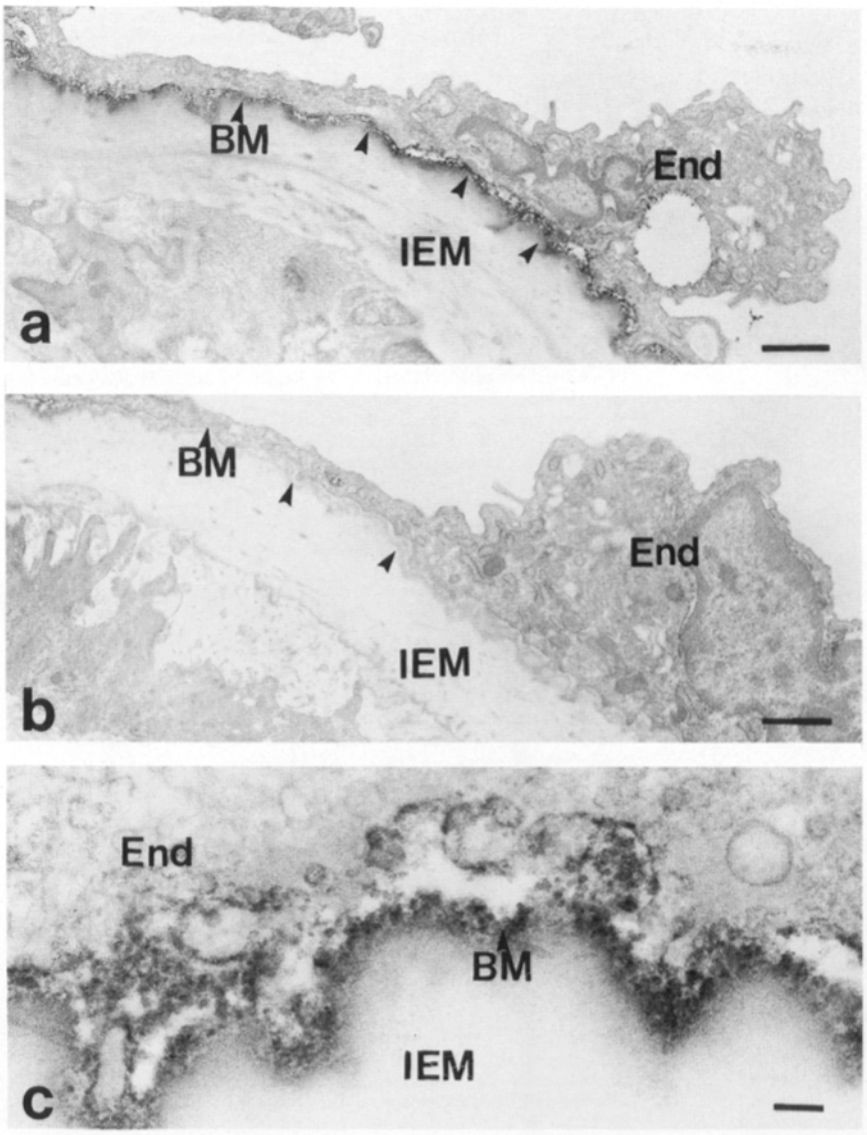

Figure 14. Electron micrographs of mouse aorta immunostained for core protein of HSPG localized on the basal lamina beneath the endothelial cells (*a*) whereas control shows no staining (*b*). Higher magnification of the basement membrane show HSPG-core localized as granules (*c*). Bars: (*a* and *b*) 1 μm; (*c*) 100 nm.

al., 1992), and core free of these chains has been observed in several tissues, including kidney and the EHS tumor (Kato et al., 1988). It has been shown that perlecan undergoes a processing to lower molecular weight forms without heparan sulfate chains in both the EHS tumor and in the glomerular basement membrane (Klein et al., 1988). Thus, it appears that a fraction of core protein, intact or processed, can be found in basement membranes.

The data support the hypothesis that the inhibiting effect of heparin is independent of heparin binding to extracellular matrix substrate but dependent upon appropriate selective site recognition. First, the ability of protein substrate to bind heparin did not correlate with the ability of heparin to inhibit cell adhesion. This makes it unlikely that the effect is mediated by a cell surface heparan sulfate which recognizes a binding site on the matrix substrate (e.g., Asch et al., 1991), or a receptor which recognizes a heparin-substrate complex. The effect here, then, appears to be different to a proposed heparin inhibition of endothelial cell spreading and chemotaxis mediated by the heparin-binding domain of thrombospondin, a cellular interaction blocked with β3 but not β1 specific antibodies (Taraboletti et al., 1990). Second, endothelial cells adhesion to a core RGD-containing synthetic peptide conjugated to BSA, although mediated by β1 and β3 class integrins, was not sensitive to heparin inhibition. Thus, even though heparin binding to substrate is not required, the cell must nevertheless recognize an appropriate determinant in the substrate for heparin to inhibit cell adhesion: this determinant is not present in an RGD-containing sequence. Whatever the actual molecular mechanism, it appears that heparin can exert its effect by either directly binding to an interacting integrin (one reason to suspect that there are two β1 integrins) or by indirectly affecting integrin function following binding to a heparin/heparan sulfate receptor. The heparin/heparan sulfate inhibition of endothelial cells adhesion to perlecan is also observed for laminin and fibronectin. The mechanisms could be similar here and glycosaminoglycans may serve a more general role in the regulation of integrin-mediated endothelial cell adhesion. This may be particularly important given the role for heparin and some heparan sulfates in the mediation of thrombosis and vascular repair.

In summary, both by specific inhibition of cell adhesion and by direct analysis of the cell surface proteins involved in core binding, we find that β1 and β3 class integrins contribute to endothelial cells adhesion. Both β1 and β3 integrins (the latter probably the vitronectin receptor) were RGD-dependent while only β1 integrin had substantial RGD-independent activity. Cell adhesion was inhibited by covalently bound or soluble heparin and heparin sulfates and these selected glycosaminoglycans may provide a specific mechanism to modulate such interactions.

We thank both Caroline Damsky and David Cheresh for generously providing blocking mAbs, and also thank Caroline Damsky for her helpful suggestions.

This study was supported by a grant-in-aid from the American Heart Association (P. D. Yurchenco, K. Hayashi) and grants RO1-DK36425 to P. D. Yurchenco and RO1-HL28373 to J. A. Madri from the National Institutes of Health.

Received for publication 24 March 1992 and in revised form 13 July 1992.

Note Added in Proof: The RGD-dependent/heparin-independent adhesion, one of the two classes of interactions identified in this study, is expected to be species-restricted because the triplet sequence is present in mouse but absent in human perlecan (Kallunki and Tryggvason, 1992; Murdoch et al., 1992).

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