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Certain High Molecular Weight Heparin Chains Have High Affinity for Vitronectin[1]

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Vitronectin is a 70-kDa protein that is found in both the extracellular matrix as well as serum. Vitronectin is one of the few proteins that regulates both the complement and the coagulation systems. Heparin is known to bind to vitronectin. Review of the literature reveals apparently conflicting outcomes of the interaction of heparin, vitronectin, and the complement system. Previous studies demonstrated that heparin diminishes vitronectin inhibition of complement activity. Numerous studies have also demonstrated that heparin exerts a net inhibitory effect on complement. We used two dimensional affinity resolution electrophoresis (2DARE) to examine this apparent paradox. 2DARE allowed simultaneous determination of binding affinity of heparin for vitronectin as well as the M_r of the heparin species. In the 2DARE experiment, the interaction of heparin with vitronectin caused retardation of the movement of the heparin through the tube gel in the first dimension. The degree of the retardation of movement was used to calculate the approximate K_d of that interaction. The heparin from the tube gel was then subjected to a second dimension electrophoresis to determine the M_r of the heparin. 2DARE analysis of the interaction of heparin with vitronectin clearly demonstrated that a sub-population of heparin chains with M_r > 8000 bound vitronectin with high affinity whereas most high M_r chains and all lower M_r chains showed little to no affinity for vitronectin. Our findings are consistent with the hypothesis that a unique binding domain exists in certain heparin chains for vitronectin.

Key Words: **vitronectin; heparin; affinity electrophoresis.**

Vitronectin, also known as S-protein (1), is a multifunctional adhesion protein present in the extracellular matrix, serum and platelets. Vitronectin has a molecular weight (M_r) of 70 kDa with a cDNA derived M_r of 42.4 kDa. An informative review of the structure and activities of vitronectin was recently published (2). Vitronectin is known to play a role in numerous biological processes including in: (1) regulation of the terminal complement pathway; (2) cellular adhesion; (3) inhibition of antithrombin III and heparin cofactor II mediated anticoagulation; and (4) regulation of plasminogen activation (2, 3). In the extracellular matrix, vitronectin interacts with a variety of structural macromolecules including heparan sulfate, dextran, and collagen. The effects of vitronectin on cell attachment and spreading were among the first functions ascribed to this glycoprotein. The interaction of vitronectin with complement is now widely recognized. Vitronectin appears to regulate the terminal pathway in two ways: (1) by interaction with soluble C5b,6,7 to promote its formation and lessen its ability to bind to membranes and (2) by preventing C9 polymerization (4). The complex interaction between vitronectin, proteoglycans (such as heparin), and complement proteins is poorly understood.

A number of studies have examined the primary structural requirements in vitronectin for heparin binding. The highly basic region of vitronectin (Ala^{341}–Arg^{379}) is important in its interaction with heparin (5). The tridecapeptide (Lys^{348}–Tyr^{360}) from this region can

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neutralize heparin binding to vitronectin (6). It now appears that the 40 amino acid cluster of basic residues on the carboxy terminal end of vitronectin, previously thought to contain the heparin binding site, may not be the only site involved in heparin binding. Phage display technology has shown that at least two regions of vitronectin are important in heparin binding, Asp^{82}–Cys^{137} and Lys^{175}–Asp^{219} (7). Fewer studies have examined the structural requirements in glycosaminoglycans for interaction with vitronectin. Some studies have examined the binding affinity of heterogenous heparin with vitronectin (8), whereas others examined vitronectin interaction with heparin analogues and cell surface glycosaminoglycans (9).

Vitronectin occurs as a monomer in circulation. This monomeric form does not bind well to heparin (10). Treatment of vitronectin with acid, urea, or heat results in enhanced heparin binding (10). Yatohgo *et al.* used this finding to demonstrate that treatment of vitronectin with 8 M urea enhances binding to heparin-agarose allowing isolation of large quantities of vitronectin from serum (11). Bittorf *et al.* demonstrated that treatment of vitronectin with >4 M urea results in a conformational change in vitronectin that causes oligomer formation and much tighter binding to heparin (12).

Several studies have examined the process by which vitronectin forms oligomers. Treatment of vitronectin with detergents, heat, extreme pH or chaotropes results in formation of oligomeric vitronectin existing as 3- to 16-mer species (13). Seiffert *et al.* demonstrated that a portion of platelet derived vitronectin exists naturally as a conformationally altered oligomer that binds heparin (14). They postulated that the heparin binding multimer of vitronectin exists in platelets due to the importance of vitronectin binding to heparin in areas of vascular injury. Zhuang *et al.* have recently reported several studies detailing the process by which vitronectin folds, unfolds, and forms multimers (15, 16). At an intermediate concentration of denaturant, an equilibrium exists between monomeric vitronectin, partially unfolded vitronectin, and fully unfolded vitronectin. The partially unfolded vitronectin readily self-associates, which is virtually irreversible at physiologic conditions (15). In a companion paper, Zhuang *et al.* showed that neither heparin nor a vitronectin derived heparin binding peptide, are able to interfere with multimerization. They concluded that the putative heparin binding site was not involved in the multimerization process (16). Zhuang *et al.* further examined the stoichiometry of the interaction of vitronectin with heparin and found the affinity of the interaction is the same for multimeric as for monomeric vitronectin when analyzed on a molar basis (8). Their study helped explain differences in the reported K_d for heparin interaction with multimeric vitronectin compared to monomeric vitronectin, by demonstrating the increased affinity for multimeric vitronectin is due to multivalent interaction with heparin rather than increased affinity of each individual interaction (8). Currently it is unknown whether triggering events occur that cause monomeric vitronectin in serum to become multimeric leading to enhanced heparin binding.

Despite the breadth and depth of reports examining the interaction of heparin with vitronectin two important questions remain. First, to what degree does cooperative binding exist in the interaction of heparin with vitronectin? Second, does the M_r of the heparin species affect binding strength? In the present study, we used 2DARE[3] to examine the interaction of heparin with multimeric vitronectin. We examined the extent to which cooperative binding and the M_r of the heparin affects the interaction.

MATERIALS AND METHODS

Materials. Outdated plasma for isolation of vitronectin was obtained from the DeGowin Blood Center, University of Iowa Hospitals and Clinics, and was used in accordance with universal precaution protocols. Sepharose 4B and heparin–Sepharose used for vitronectin purification and CHAPS were purchased from Sigma Scientific (St. Louis, MO). Antibody to human vitronectin was purchased from Quidel (San Diego, CA). Western blot analysis was conducted as described (17) with materials from Bio-Rad (Hercules, CA). Capillary tubes (150 mm), ammonium hydroxide, sodium acetate, and sodium citrate were from Fisher Scientific (Fair Lawn, NJ). Glacial acetic acid was from Mallinckrodt (Paris, KY). The first dimension electrophoresis unit, Hoefer SuperSub Horizontal Gel Electrophoresis Unit, Model HE 100, was from Hoefer Scientific (San Francisco, CA). Low melting point agarose, acrylamide, ammonium persulfate, boric acid, glycine, piperazine diacrylamide, Tris (hydroxymethyl) aminomethane, Protean II xi Multi-Gel Casting Chamber, and Protean II xi Multi-Gel Electrophoresis Unit were from Bio-Rad. Porcine mucosal heparin, (163 units/mg) and Bovine Serum Albumin (BSA) were from Kabi Pharmacia (Franklin, OH) and has been analyzed in detail by our laboratory (18). The partially heparin lyase depolymerized bovine lung heparin has also been previously characterized by our laboratory (19, 20). Alcian yellow, silver nitrate, 1,4-dimethylpiperazine, and sodium thiosulfate were from Aldrich Chemical (Milwaukee, WI). Bromophenol blue was from MCB Manufacturing Chemists (Cincinnati, OH). Disodium ethylenediamine tetraacetate and sucrose were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Methanol was from EM Science (Gibbstown, NJ).

Purification of vitronectin. Vitronectin was isolated from aged citrated human plasma using the method of Yatohgo *et al.* (11). Briefly, plasma was clotted by addition of $CaCl_2$ and then applied to a Sepharose 4B precolumn followed by a heparin-Sepharose column. Flow through was treated with urea and then reapplied to the heparin-Sepharose column. Pure vitronectin was eluted from the column. Purity was demonstrated by polyacrylamide gel electrophoresis and Western blot analysis as previously described (17).

[3] Abbreviations used: 2DARE, two-dimensional affinity resolution electrophoresis; BSA, Bovine Serum Albumin; Buffer A, 50 mM, 3-{*N*-morpholino}-2-hydroxypropanesulfonic acid, sodium salt, 125 mM sodium acetate, pH 7.4; CHAPS, 3-{(3-cholamidopropyl)-dimethylammonio}-1-propane-sulfonate, 3-{*N*-morpholino}-2-hydroxypropanesulfonic acid; K_d, dissociation constant; m, heparin mobility; m_f, mobility of "free" (nonbound) heparin; M_r, average molecular weight; R, retardation coefficient $\{R = (m_f - m)/m_f\}$.

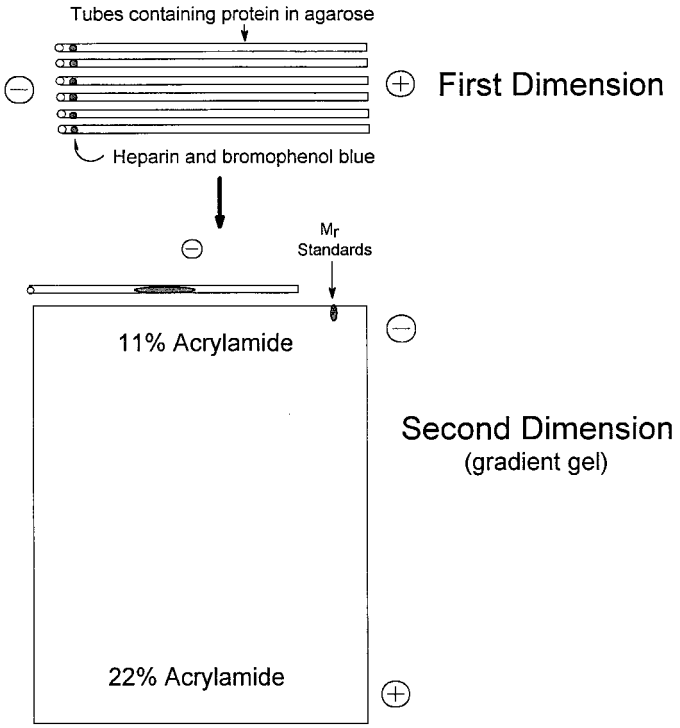

FIG. 1. The 2DARE method. In the first dimension of the analysis, heparin is moved by electrophoresis through varied concentrations of vitronectin (0–19 μM) dissolved in 1% agarose. The tube gels are extruded onto the edge of a gradient polyacrylamide gel for the second dimension analysis. The gels are stained with alcian yellow followed by silver staining to detect the heparin. Depolymerized heparin acting as a M_r standard is included on each gel and is used to determine M_r of the experimental heparin.

Various amounts of purified vitronectin (150–450 ng) as well as M_r standards were subjected to polyacrylamide gel (10%) electrophoresis followed by silver staining of one gel and Western blot analysis of the other.

2DARE analysis of vitronectin interaction with heparin. The 2DARE method (21) was used to analyze the interaction of vitronectin with commercial heparin (summarized in the legend of Fig. 1). Briefly, various amounts of vitronectin (0–19 μM) were dissolved in 1% agarose/0.5% CHAPS in Buffer A. Separate capillary tubes were filled with each of the vitronectin/agarose dilutions. After the agarose gelled, heparin (1 μg) was added to one end of the tube (the origin). The heparin had been dissolved in Buffer A (0.1% Bromophenol blue tracking dye and 6% sucrose). Next, 3–5 μl 0.5% agarose in Buffer A (65°C) was layered over the heparin to prevent the heparin from leaking from the end of the tube when laid horizontally. The tube gels were placed in the first dimension electrophoresis unit and submerged in Buffer A (4°C). Electrophoresis was conducted at constant voltage (55 V) using an ISCO model 453 power supply for 4 h. During electrophoresis, heparin (negatively charged) moved through the vitronectin containing gel toward the positive electrode. A control experiment was conducted in which 1 μg heparin was moved through 5 mg/ml BSA under the same conditions as described for heparin through vitronectin.

Gradient polyacrylamide gels for the second dimension were prepared as described previously (21). Gels were simultaneously cast in the Multi-Gel Casting Chamber. A 5% polyacrylamide stacking gel was added to the top of each gradient polyacrylamide gel (5% polyacrylamide in 100 mM boric acid, 100 mM tris (hydroxymethyl) aminomethane, 10 mM disodium ethylenediamine tetraacetate, 27 mM 1,4-dimethylpiperazine, 0.04% ammonium persulfate, pH 6.3, filling approximately 1 cm). The tube gels were carefully laid on the stacking gel so that the tube gel origin was exactly lined up with one edge of the polyacrylamide gel. Partially, heparin lyase-depolymerized bovine lung heparin (300 ng in Buffer A that was 0.1% bromophenol blue and 6% sucrose) was used as a M_r standard (a "ladder"), to allow subsequent determination of M_r of heparin species (20). The polyacrylamide gels were fitted to the electrophoresis cooling cores and placed in the lower chamber buffer (100 mM boric acid, 100 mM tris (hydroxymethyl) aminomethane, 10 mM disodium ethylenediamine tetraacetate, pH 8.3). Buffer (0.2 M Tris (hydroxymethyl) aminomethane, 1.25 M glycine, pH 8.3) was added to the upper chamber of each cooling core and electrophoresis was conducted at 4°C and at a constant current of 6 mA/gel for 32 h.

The polyacrylamide gels were stained with 0.5% alcian yellow in 30% acetic acid for 20 h. Excess alcian yellow was removed by soaking in 10% acetic acid, then 50% methanol, and then distilled water with washes. The alcian yellow stained heparin in the polyacrylamide gels was silver stained using ammoniacal silver as described previously (21). Development occurred within 7–12 min. After developing, the gels were placed in a stop solution (30% methanol, 10% acetic acid) for 1 h and stored in distilled water until photographed at 1:1 image size.

Determination of the M_r of heparin chains. The M_r of each heparin chain visible on the gradient gels was determined as previously described (20). When heparin lyase-digested heparin moved in an electrophoretic field into a gradient polyacrylamide gel, a "ladder" forms. The primary band that moves the farthest into the gel is two sugar residues in length. The ladder increases progressively by two sugar residues. The number of sugar residues represented by each band can be used to calculate M_r. It has been shown previously that the log of the M_r is linearly related to the distance of migration of the band into the gradient polyacrylamide gel (20). Thus, the distance the heparin lyase-digested heparin migrated into the second dimension gel was used to construct a standard curve of log of M_r vs distance migrated. This curve was then used to predict the M_r of each heparin band that had been subjected to interaction with vitronectin.

Determination of heparin affinity for vitronectin. The migration distances of the leading and tailing edge of each heparin band for a given M_r was measured from 1:1 photographic images of the 2DARE gels. For a given M_r, each distance that part of the heparin band migrated in the first dimension was divided by the electrophoresis time (7.3 h) to calculate m, heparin mobility. The data obtained from these distances were used to calculate $1/m\text{-}m_f$, where m_f = mobility of "free" (nonbound) heparin. These data were then used to calculate the retardation coefficient, R using the formula $R = (m_f - m)/m_f$ (22, 23). The data were fitted to the equation $R = R_{oo}/(1 + K_d/\{\text{vitronectin}\}^n)$, using the nonlinear least-squares fitting program NONLIN (Michael Johnson, University of Virginia Health Sciences Center, Charlottesville, VA).

RESULTS

The vitronectin purified from plasma was subjected to SDS–polyacrylamide gel electrophoresis with silver staining as well as Western blot analysis. The silver staining demonstrated a single band with a M_r of ~70 kDa as shown in Fig. 2. The vitronectin was then subjected to Western blot analysis as previously described (17), which confirmed that the protein isolated was human vitronectin (not shown).

Heparin (1 μg per tube gel) was subjected to the first dimension of the electrophoresis analysis. Four representative gels obtained from the second dimension are

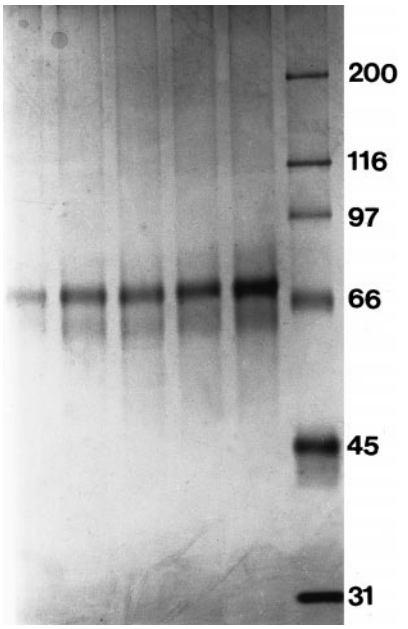

FIG. 2. Demonstration of the purity of the isolated vitronectin. Various amounts of vitronectin (150–450 ng) and M_r standards were subjected to polyacrylamide gel electrophoresis and silver staining. A single band with a M_r near 70 kDa is seen.

shown in Fig. 3 (panel 1). The origin of the tube gel is on the far left of each second dimension gel. Movement of heparin through the first dimension gel is in the direction indicated by the arrow on gel B (grid is cm moved). Gels C and D appear to have diminished levels of lower M_r chains which is a photographic artifact. On the actual experimental gels, the M_r range of the heparin is identical on all gels. On the far right of each gel are the heparin derived M_r standards with size (numbers of sugar residues) labeled on gel B. The standards show up to varying degrees on the photographic copies, but are easily seen on the actual gels. Heparin appears as darkly stained bands of varied widths. The gels shown differ by the vitronectin concentration used in first dimension: A = 12.9, B = 10.0, C = 7.2, and D = 0 μM vitronectin. A total of eight concentrations of vitronectin (0–19 μM) were used (the other four are not shown). This range of vitronectin concentrations was chosen after a number of preliminary experiments had shown that below 2 μM vitronectin, the movement of heparin was not altered. When the concentration of vitronectin is above 30 μM in agarose, the agarose will not gel making the experiment impossible. In Fig. 3 (panel 2) we show the results of the control experiment where heparin was moved through 5 mg/ml BSA (upper gel) and compared with heparin moved through agarose alone (lower gel). As shown, no retardation of the movement of heparin was seen, indicating no interaction between heparin and BSA.

Mathematical calculations of experimental reproducibility are not provided because this type of experiment does not lend itself to a reliable calculation of reproducibility. The primary data obtained from each gel is distance of heparin movement. Experiments done at different times would have different distances traveled for a given vitronectin concentration simply based on time of the electrophoresis, resistance of the system and voltage. In fact, each experiment is effectively "self-controlled" due to the fact that heparin movement through vitronectin is compared with its movement through no protein. Altogether, the heparin through vitronectin experiments were conducted >10 times with four different vitronectin preparations. The pattern of retardation of high M_r heparin movement through high concentrations of vitronectin compared to less retardation of movement at lower vitronectin concentrations was highly reproducible.

The M_r of various heparin chains shown in Fig. 3 were determined. A plot of the log M_r versus the distance the standards moved into the gradient polyacrylamide gel is shown in Fig. 4. As shown, the plot of log M_r versus distance traveled is linear and has a correlation coefficient (r^2) of 0.99. From this line, we were able to determine the M_r of various heparin chains on the gels shown in Fig. 3 by measuring the distance migrated into the gradient gel.

Examination of gel D in Fig. 3 (heparin through no vitronectin) reveals a 2-cm-wide band which ranges in M_r from about 1,500 to over 15,000. On gels A through C (gels with varied amounts of vitronectin) there is a broadening of the heparin band in the horizontal plane (left to right on the gel). This results from the retardation of the movement of some heparin chains as they traversed the vitronectin containing agarose gel. The most retarded heparin appears to have had the highest M_r, whereas the least retarded heparin had the lowest M_r of the group of heparin chains whose movement was retarded (especially well seen on gel A). In addition, some of the high M_r heparin chains did not appear to be retarded by presence of vitronectin (see especially gel C), indicating selective interaction of certain high M_r chains with vitronectin. Furthermore, the minimal length of heparin that had its movement retarded by the presence of vitronectin was 26 sugar residues, corresponding to a M_r of about 8200.

The migration distances of the bound (m) and free (m_f) heparin were used to calculate the retardation coefficient as described above. The data were fitted to the equation $R = R_{oo}/(1 + K_d/\{\text{vitronectin}\}^n)$, using the nonlinear least-squares fitting program NONLIN for n = 1, 3, 5, 6, and 12 and plots obtained. The smooth curves obtained represent the fits at varied values of the n-term (n = 1, 3, 5, 6, and 12) and are shown in Fig. 5. The curves appear to fit the data better as the values of the n-term increase. This suggests that vitronectin

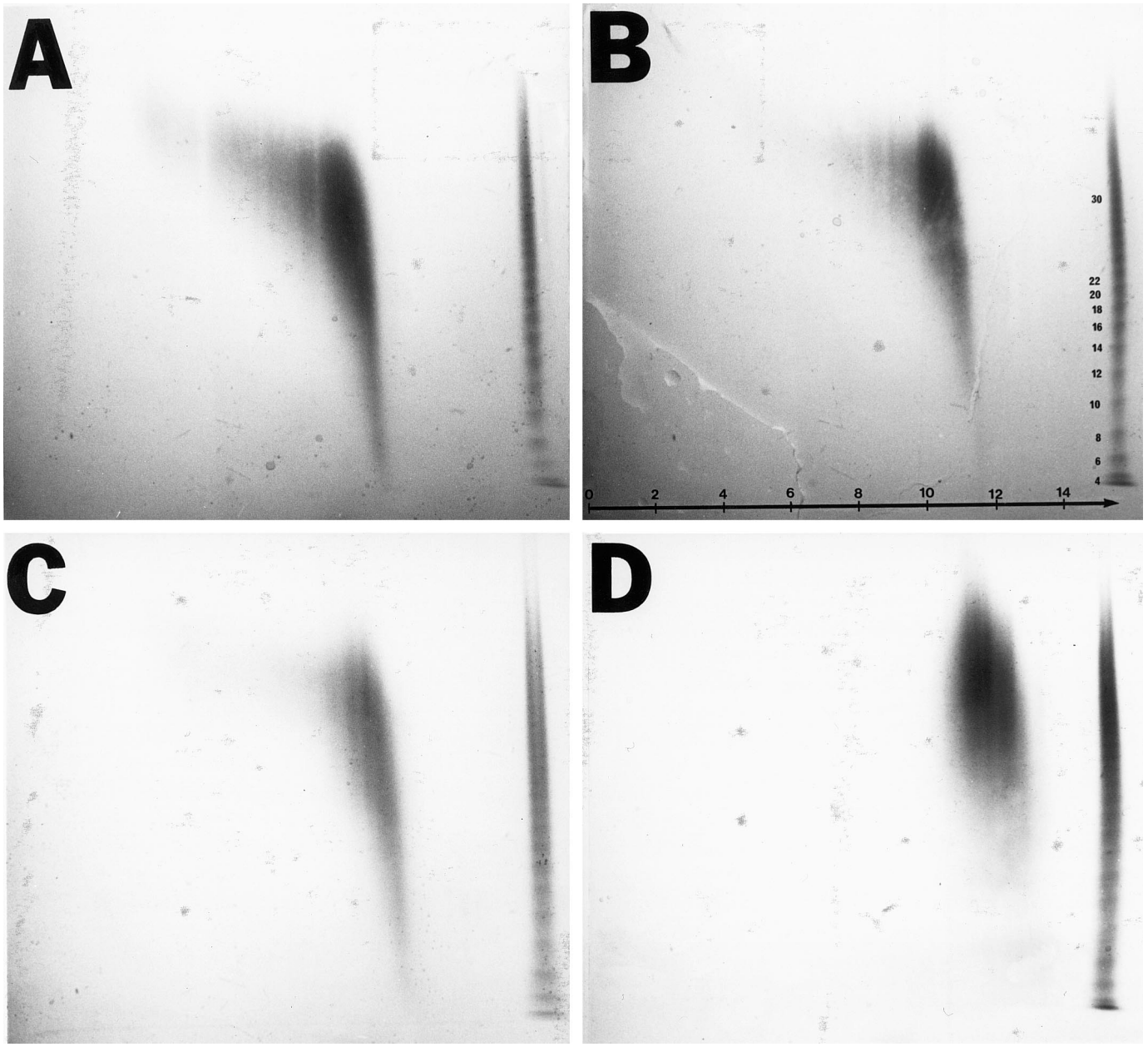

FIG. 3. 2DARE analysis of heparin interaction with vitronectin. In the first dimension, 1 μg heparin was moved through a series of 1% agarose tube gels containing 0–19 μM vitronectin. The tube gels were then placed on the top edge of the second dimension gradient polyacrylamide gel and subjected to electrophoresis. The second dimension gels were then stained with alcian yellow followed by silver staining. In Panel 1, four representative second dimension gels are shown labeled A–D corresponding, respectively, to 12.9, 10.0, 7.2, or 0 μM vitronectin in the first dimension tube gel. On gel B, M_r standards are labeled by number of sugar residues. The grid and arrow shows the direction of heparin movement in the first dimension and distance (cm) migrated. Comparing gels A and B (12.9 and 10.0 μM vitronectin) with gel D (no vitronectin) reveals that a fraction of high M_r heparin chains were significantly retarded when moving through vitronectin in the first dimension. Panel 2 is the 2DARE control experiment. This control was used to demonstrate that the retardation of heparin movement shown in Panel 1 is not an artifact. In this experiment, 1 μg heparin was also moved through 5 mg/ml BSA (Panel 2, upper gel) or no protein (Panel 2, lower gel) in the first dimension agarose tube gel and then subjected to the second dimension electrophoresis in the same manner as described for vitronectin in Panel 1. The resulting second dimension gels are shown in Panel 2 and shows no difference in heparin movement through BSA compared with heparin movement through agarose containing no protein.

interaction with heparin is cooperative (22, 23). When the three terms (R_{oo}, K_d, and n) were allowed to float, the values 0.784, 8.53×10^9 μM, 12.2 were determined, respectively. The n-th root of the calculated K_d value gives an apparent K_d value of 6.7 μM, at which binding is half-maximal.

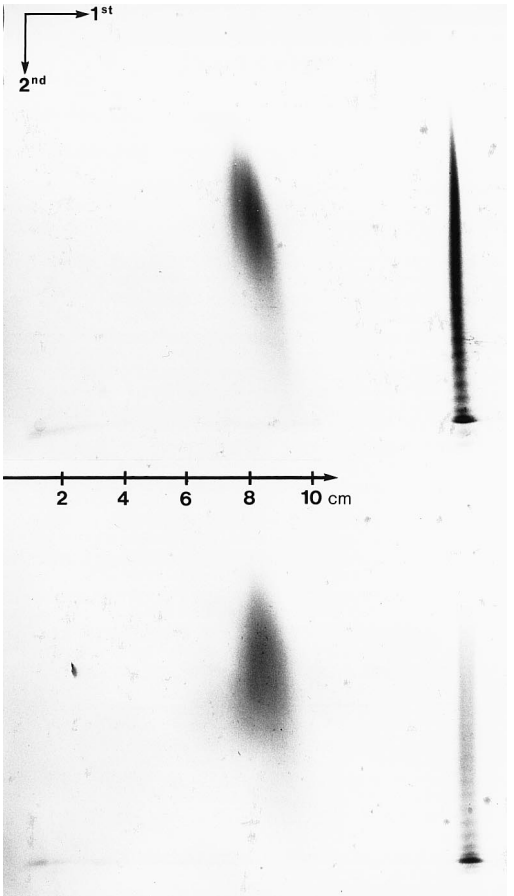

FIG. 3—*Continued*

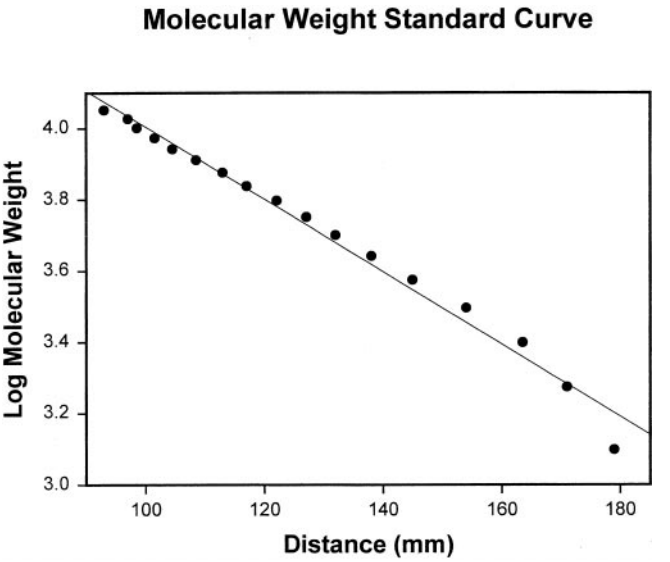

FIG. 4. M_r standard curve. For each 2DARE gel (as shown in Fig. 3), the distance each M_r standard band moved into the second dimension gel was measured and plotted against the log of each M_r. As shown, a linear relationship exists between M_r and the distance moved. Plots were used to calculate M_r of various heparin chains obtained in the 2DARE analysis of heparin interaction with vitronectin shown above.

DISCUSSION

This study used 2DARE analysis to demonstrate that a relatively small number of high M_r heparin chains bound tightly to vitronectin ($K_d \sim 6.7$ μM). Previously reported K_d for the interaction of heparin with vitronectin range from 4–40 μM with most authors reporting K_d values near 5 μM (6, 8, 24, 25). It has been shown that the K_d for this interaction differs depending on the character of the isolated vitronectin. Specifically, urea-treatment during the purification of vitronectin results in oligomer formation, which binds heparin more readily than does native or monomeric vitronectin (12). It has also been shown that multimeric vitronectin occurs naturally in platelets raising the specter that events occur in vascular injury that trigger the conformational change necessary to allow multimeric vitronectin to form rendering it capable of binding heparin (14). We purified vitronectin using urea treatment (11) to enable isolation of multimeric vitronectin with the greatest affinity for heparin. Recent studies have explored the role of cooperative binding in the interaction between heparin and vitronectin (25). Our goal was to examine both the affinity of heparin for oligomeric vitronectin and to determine if certain heparin chains exist with high affinity for vitronectin that would support the hypothesis that heparin contains discrete binding domain(s) for vitronectin.

The 2DARE method allows easy visualization of multiple heparin chains with varied affinity for vitronec-

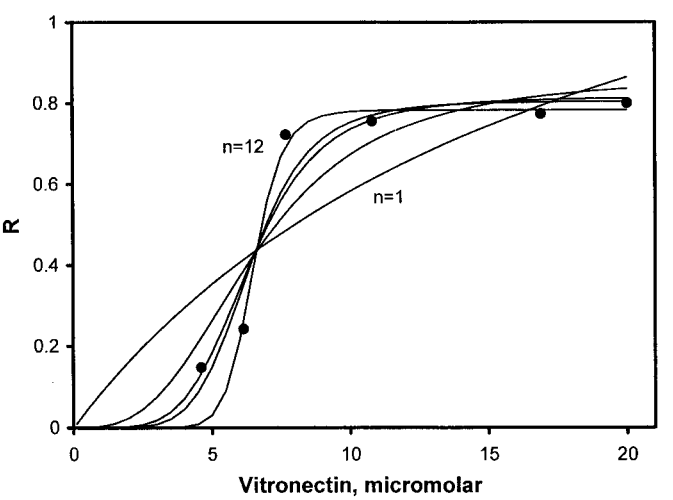

FIG. 5. Binding curves for heparin interaction with vitronectin. Vitronectin concentration versus R were plotted. Data were fit to the equation $R = R_{\infty}/(1 + K_d/\{\text{vitronectin}\}^n)$ using a NONLIN program. Curves obtained represent fits at varied values of n (1, 3, 5, 6, and 12.2). The curve fit to the data improves an n increases with the best fit obtained at $n = 12.2$.

tin. Specifically, we showed that the majority of the high M_r heparin chains present in commercial heparin do not bind to vitronectin. However, a fraction of high M_r heparin chains does bind tightly to vitronectin as demonstrated by the significant retardation of those heparins when moving through the vitronectin containing agarose gels. Interestingly, a minimum chain length of 26 sugar residues in heparin (M_r approximately 8200) is necessary for tight binding to vitronectin. The high minimum chain length required for tight binding may indicate either that: (1) two binding domains exist on the heparin chain that are necessary for binding to vitronectin, or (2) a single binding domain exists that is readily destroyed as heparin degrades or is catabolized, or (3) a rare binding domain exists. This finding may be of significant clinical relevance considering the widespread use of low molecular weight heparins (average M_r = 5–8 kDa) (26). Varki *et al.* demonstrated that unlike unfractionated heparin, low M_r heparins fail to inhibit L- and P-Selectins (27). They postulate that this might explain the decreased anti-inflammatory activity of low M_r heparins compared with unfractionated heparin. Our results strongly support the prediction that low M_r heparins can have activities distinct from unfractionated heparin. To answer these questions will require a much more extensive understanding of the structure–activity requirements present in heparin for interaction with vitronectin and other proteins.

To analyze the affinity of heparin for vitronectin, a variation of affinity electrophoresis was used. When two ligands are examined in an affinity electrophoresis system, changes in mobility are related to the variation of the ligand concentration. In the present study, the degree to which any given heparin chain had its movement retarded while passing through vitronectin can be correlated with affinity of that particular heparin chain for vitronectin. Shimura derived equations that enable shifts in electrophoretic mobility of the detected molecule to be plotted as a function of the varied concentration of the other molecule (28). According to Shimura, a plot of $1/m\text{-}m_f$ (essentially the degree of retardation of the movement of heparin through the first dimension gel) versus 1/vitronectin concentration is a straight line. The plot can be used to calculate the K_d of the interaction as the x-axis intercept is equal to $-1/K_d$.

In the present study, we found that the plot of R versus vitronectin concentration did not yield a straight line. In fact, the resulting curve is clearly sigmoidal shaped. Thus, we used a nonlinear least-squares fitting program to fit the data to the equation $R = R_{oo}/(1 + K_d/\{\text{vitronectin}\}^n)$ as previously described (22, 23). The values obtained for R, K_d and $\frac{1}{2}$ maximal K_d are summarized in Table I. As the plot of the resultant data shows (Fig. 5), the data fit improves significantly with an increase in the value of n with n = 12, allowing the best fit of the data to the curve. This is compelling evidence that significant cooperative binding exists in the interaction of vitronectin with heparin. Our findings are strongly supportive of the work by Gibson *et al.* that used fluorescence spectroscopy to document cooperative binding between heparin and vitronectin (25). Thus, our data help explain some of the apparent discrepancies in the literature with respect to the K_d of this interaction. Furthermore, our findings support the assertion by Liang *et al.* that two or more binding sites exist on a given heparin chain for vitronectin (7).

TABLE I

K_d for Varied n Determined Using NONLIN Program to Best Fit Curves Obtained from Fig. 5 to the Equation $R = R_{oo}/(1 + K_d/\{\text{Vitronectin}\}^n$

R	n[b]	K_d (μM)	nth root $\frac{1}{2}$ maximal K_d (μM)[a]
1.67	1	18.6	18.6
0.96	2	53.5	7.30
0.86	3	287	6.59
0.83	4	1,726	6.44
0.81	5	10,785	6.40
0.805	6	69,381	6.41
0.784	12.2[c]	8.53×10^9	6.72

[a] The nth root of the calculated K_d value is reported to indicate the protein concentration at which binding is half maximal.
[b] The n term value was fixed for all except n = 12.2.
[c] The three terms (R_{oo}, K_d, and n) were allowed to float in the fit.

Unfortunately, the technology to quantify precisely the portion of heparin chains that interact with vitronectin from the total pool of heparin chains is not readily available. However, when examining the gels presented here, one can estimate this proportion by visual analysis. As shown in Fig. 3, clearly over half of the heparin chains do not bind to vitronectin (migrate essentially at the same rate as heparin through agarose alone). We would estimate that only 20–30% of the total number of heparin chains have their migration retarded by movement through the vitronectin containing gels. This observation would strongly support the theory that specific binding sites for vitronectin exist on certain heparin chains, but are relatively rare.

The present study examined the interaction of heparin with vitronectin using a two-dimensional affinity electrophoresis method. We found that heparin interacts with vitronectin with an affinity near 6 μM. We also found that a significant degree of cooperative binding exists in this interaction. Furthermore, we demonstrated that the minimum heparin chain length required to achieve that degree of binding is approximately 26 sugar residues. The fact that less than half the higher M_r heparin chains appear to bind to vitro-

nectin strongly supports the theory that a specific binding site or sites exist in heparin for vitronectin.

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