New insights into heparin binding to vitronectin: studies with monoclonal antibodies

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Vitronectin is a plasma glycoprotein that binds to a variety of ligands. There is considerable debate regarding the dependency of these binding interactions upon the conformational status of vitronectin, the role of multimerization and how the binding of different ligands can change vitronectin's conformational state. We have developed a method of capturing vitronectin directly from fresh plasma using solid-phase monoclonal antibodies. Various biotin-labelled secondary monoclonal antibodies were used to quantify the bound vitronectin and to measure its degree of denaturation. Using these tools we demonstrated that one monoclonal antibody partially denatured vitronectin without direct multimerization. Treatment of vitronectin in plasma with soluble heparin produced a similar degree of denaturation. These

results led to a proposed adaptation of the unfolding/refolding pathways for chemically denatured vitronectin originally presented by Zhuang and co-workers in 1996 [Zhuang, Blackburn and Peterson (1996) *J. Biol. Chem.* **271**, 14323–14332 and Zhuang, Li, Williams, Wagner, Seiffert and Peterson (1996) *J. Biol. Chem.* **271**, 14333–14343]. The adapted version allows for the production of a more stable partially unfolded intermediate, resulting from the binding of particular ligands. We also demonstrated that the avidity of heparin binding to vitronectin is governed by both the conformational state of the monomer and multimerization of the molecule.

Key words: conformation, ligand binding.

INTRODUCTION

Vitronectin is a 75 kDa glycoprotein that circulates in mammalian plasma as a folded monomer at a concentration of approx. 400 µg/ml [1]. It is also found in the extracellular matrix in an unfolded multimeric form. It is capable of binding a wide variety of ligands and there has been considerable argument in the literature regarding the conformation dependency of these interactions. This is coupled with the argument regarding the number and nature of conformational states of vitronectin and how the binding of different ligands can change its conformational state. The two extremes in vitronectin conformation are probably represented by the native monomeric molecule in plasma, which exhibits minimal ligand reactivity, on the one hand, and multimeric vitronectin, which can be induced by exposure to (and subsequent removal of) strong denaturing agents, such as 8 M urea, low pH or high heat, and exhibits a high degree of ligand binding, on the other [2].

One of the ligands in question is heparin. Early studies on the binding of vitronectin from fresh plasma to heparin–Sepharose reported less than 5% binding [3,4]. Conversion of plasma to serum increased the binding activity of vitronectin to heparin–Sepharose, which reached 25% in some reports [3–5]. Storage of frozen plasma has also been reported to result in increased binding to heparin–Sepharose [3].

In contrast to the above weak binding reactions between native plasma vitronectin and heparin–Sepharose, there is compelling evidence that plasma vitronectin binds free heparin in solution. Vitronectin purified from plasma, using methods which

did not involve ligand binding or denaturants, was shown to inhibit the heparin-mediated catalysis of antithrombin III inhibition of thrombin and factor Xa [6,7]. The latter reaction was also demonstrated to take place with native vitronectin in plasma. More recent studies have shown that vitronectin in plasma quantitatively forms stable complexes with heparin [8].

The apparent contradiction regarding the ability of native plasma vitronectin to bind to heparin lies in the types of assay used to measure it. There are many heparin binding proteins present in plasma and their interaction with heparin bound to Sepharose is likely to be both time- and concentration-dependent. Under these conditions vitronectin monomer, which has a relatively low affinity for heparin compared with several other plasma proteins, may bind poorly to heparin–Sepharose [8]. Also, if one of the species (heparin in this case) is bound to a solid-phase support, the flexibility of the molecule is restricted and this may reduce the operative affinity of the binding reaction [8].

Multimeric vitronectin has been demonstrated in numerous cases to bind to heparin in solid-phase assays with efficiency that is orders of magnitude higher than purified native vitronectin or native vitronectin in plasma [2,4,9–12]. It was concluded in the earlier studies that greater binding of the vitronectin multimer was due to altered conformation of the component monomers to expose an erstwhile obscured or restricted heparin-binding site. More recent studies by Zhuang et al. [13] of the binding of purified monomeric and multimeric vitronectin to heparin in solution suggested that the intrinsic affinity between binding sites was not significantly different in the two cases. The increased

Abbreviations used: mAb, monoclonal antibody; ABTS, 2,2'-azino-bis(3-ethylbenzthiazole-6-sulphonic acid); 13K heparin, 13 kDa heparin; 6K heparin, 6 kDa heparin; Bi, biotinylated; BiHep/BSA, heparin conjugated to biotinylated BSA; NHS-biotin, *N*-hydroxysuccinimido-biotin; SNK, Student–Newman–Keuls; PAI-1, type 1 plasminogen activator inhibitor.

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binding to vitronectin multimer was postulated to be due solely to the increase in avidity brought about by multimerization increasing the stoichiometry of the reaction, particularly for higher-molecular-mass heparins which can accommodate more than one vitronectin molecule. The preparation of purified vitronectin used in these studies, however, involved an affinity-binding step on heparin–Sephadex at low pH. Binding under these conditions produces monomeric vitronectin but whether or not the conformation is truly native is unknown. Furthermore, the heparin-binding reaction was also studied in a low salt buffer, so the interpretation of the findings in a physiological context is suspect. A more recent study by Francois et al. [14] of heparin binding to native vitronectin monomer in solution was unable to demonstrate binding. Although the information in this report suggested that physiological saline was used as a diluent, this was not stated definitively.

We have developed a method of studying ligand binding of plasma vitronectin by capturing it directly on solid-phase-bound monoclonal antibodies (mAbs). This avoids any inadvertent denaturation of vitronectin during the purification steps and therefore utilizes vitronectin in a state as close to native as is possible to achieve. This can be monitored by use of a separate mAb whose binding is exquisitely sensitive to the degree of denaturation of vitronectin. Using this system we have further investigated the binding of heparin to vitronectin and demonstrate that the avidity of the reaction is governed by both the conformational state of the monomer and multimerization of the molecule.

MATERIALS AND METHODS

Materials

N-hydroxysuccinimido-biotin (NHS-biotin) was from Bio-Rad Laboratories, Regents Park, NSW, Australia. Horseradish peroxidase conjugated to streptavidin was from Amersham Bioscience, Melbourne, Australia. Horseradish peroxidase conjugated to rabbit anti-mouse IgG was from Dako. Peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazole-6-sulphonic acid) (ABTS) and heparin sodium salts of average molecular masses 13 and 6 kDa (H-3149 and H-5284 respectively; referred to as 13K heparin and 6K heparin) were from Sigma-Aldrich (Castle Hill, NSW, Australia). They were dissolved in PBS, pH 7.1, at a concentration of 10 mg/ml and stored at -20°C . Before use the heparin was boiled for 2 min. 13K Heparin conjugated to biotin-labelled albumin (H-4016) was also from Sigma-Aldrich. This reagent had a coupling ratio of 3–6 molecules of heparin/molecule of albumin. It was dissolved in PBS at 1 mg/ml of protein and stored at -70°C in small aliquots. All other chemicals were of analytical grade. ELISA plates were poly(vinyl chloride) U-bottomed microtitre plates from Dynex Technologies, Chantilly, VA, U.S.A.

Vitronectin preparations

Platelet-poor plasma was prepared from freshly collected citrated human blood by centrifugation at 2000 *g* for 30 min. The plasma was stored in aliquots at -70°C . Vitronectin was purified from fresh plasma by double passage over an affinity column of mAb HV2 (see below) coupled to CNBr-activated Sepharose (Amersham Bioscience), and eluted with 3 M MgCl_2 as previously described [15]. Purity of the product was checked by SDS/PAGE under reducing conditions. On occasion, mAb HV2 leached off the Sepharose column together with eluted vitronectin. This contaminating mAb was removed by passage over a Protein A–Sepharose Hi-Trap column (Amersham Bioscience).

Vitronectin purified in this manner is referred to as HV2-purified vitronectin. It was stored in PBS at pH 8.0 in small aliquots at -70°C in siliconized vials. Denatured vitronectin was prepared by incubation of HV2-purified vitronectin made to 8 M urea for 2 h at room temperature, followed by extensive dialysis against PBS at pH 8.0. Denatured vitronectin was stored in small aliquots at -70°C . The distribution of molecular species of vitronectin in these preparations was tested by non-denaturing electrophoresis [16] on 4–20% gradient acrylamide Tris/glycine minigels (Gradipore, Sydney, NSW, Australia) in a Bio-Rad Mini Protean II electrophoresis system. Separated proteins were blotted on to Immobilon-P PVDF membranes (Millipore, Bedford, MA, U.S.A.) by the method of Towbin et al. [17] in a Bio-Rad transfer cell. Transferred vitronectin was detected using mAb HV2 and peroxidase-conjugated rabbit anti-mouse IgG, followed by 4-chloro-1-naphthol as a substrate (Sigma-Aldrich).

mAb preparations

mAbs to human vitronectin, HV1, HV2, HV3, HV6, HV8, HV11, HV16, HV21 and HV23, have been described previously [18]. mAbs HV1, HV2 and HV8 were labelled with biotin by incubation at 1 mg/ml with a $50\times$ molar excess of NHS-biotin in 0.1 M bicarbonate buffer, pH 8.1, for 10 min in the dark. The reaction was quenched with excess NH_4Cl and unbound biotin was removed by buffer exchange into 0.1 M bicarbonate, pH 8.1, using Centricon 30s (Amicon, Beverly, MA, U.S.A.). The biotinylated mAbs were stored in small aliquots at a concentration of approx. 1 mg/ml at -70°C . The biological activity of the labelled mAbs was checked by comparative dilution curves with unlabelled mAbs, in a standard ELISA using coated denatured vitronectin, peroxidase-conjugated rabbit anti-mouse IgG (1/1000 dilution) and ABTS substrate. No loss of biological activity was observed with any of the labelled mAbs. Biotinylated (Bi) HV2 and HV8 were subsequently used to quantify bound vitronectin. These two reagents were titrated in a checkerboard fashion against a dilution series of plasma, HV2-purified vitronectin or denatured vitronectin coated on ELISA wells. Working concentrations of the biotinylated mAbs were chosen within the range that displayed linear binding curves for sub-saturation coating concentrations of vitronectin (i.e. not mAb-limited). Within this range, concentrations of each labelled mAb were chosen so that they each gave similar absorbance readings for similar surface concentrations of vitronectin. For BiHV2 this was 4 $\mu\text{g}/\text{ml}$ and for BiHV8 1 $\mu\text{g}/\text{ml}$. These two reagents could be used interchangeably to quantify vitronectin bound to other solid-phase mAbs (see below). BiHV1 produced lower absorbances than BiHV2 or BiHV8 and was used at 4 $\mu\text{g}/\text{ml}$.

Vitronectin peptides

In previous studies we had determined that the mAb panel bound to vitronectin epitopes located in CNBr fragments contained between amino acids 1 and 310 [18]. Utilizing the sequence data reported by Yoneda et al. [19], we determined 11 discrete sites of sequence variation between the human and mouse forms of this region of vitronectin. Large continuous sections of the connecting region (amino acids 50–130) displayed sequence variation and discrete sites could not be separated. The following peptides from the human sequence were synthesized from the discrete regions (amino acids are represented by standard single-letter code, and positions of variation from the mouse sequence are shown in *italics*): AU16, amino acids 9–18, CTEG**FN**VDKK; AU17, amino acids 33–43, TDY**TA**ECKPQV; AU18, amino acids 126–135, GR**PQ**PPAE**EE**; AU19, amino acids 164–173, LDEKAVRPGY; AU20, amino acids 175–184, KLIRDV-

WGIE; AU21, amino acids 216–225, LDPDYPRNIS; AU22, amino acids 224–234, ISDGFDGIPDN; AU23, amino acids 236–249, DAALALPAHSYSGR; AU24, amino acids 262–271, EYQFQHQPSSQ; AU25, amino acids 287–296, AMMQRDSWED; AU26, amino acids 305–315, GRTSA-GTRQPPQF.

Overlapping peptides were synthesized from the connecting region as follows: AU29, amino acids 55–64, EYTVYDDGEE; AU30, amino acids 65–74, KNNATVHEQV; AU31, amino acids 75–84, GGPSLTSDLQ; AU32, amino acids 85–94, AQSKGNPEQT; AU33, amino acids 95–103, PVLKPEEEA; AU35, amino acids 104–112, PAPEVGASK; AU28, amino acids 113–124, PEGIDSRPETLH; AU36, amino acids 60–69, DDGEEKNNAT; AU37, amino acids 70–79, VHEQVGGPS; AU38, amino acids 80–89, TSDLQAQSKG; AU39, amino acids 89–98, GNPEQTPVLK; AU40, amino acids 99–108, PEEAPAPEV; AU41, amino acids 109–118, GASKPEGIDS; AU42, amino acids 121–133, ETLHPGRPQPPAE. Peptides were synthesized on a PerSeptive Pioneer Peptide Synthesis System, using fluorenylmethoxycarbonyl/2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/*N*-hydroxybenzotriazole (Fmoc/HBTU/HOBt) chemistry and Fmoc/Tris(alkoxy)benzylamine/poly(ethylene glycol)/polystyrene (Fmoc-PAL-PEG-PS) resin. All N-termini were acetylated and C-termini amidated; 5 mg of each peptide was dissolved in 100 µl of DMSO. This was increased to 1 ml with distilled water. Peptide stocks were stored in aliquots at –70 °C.

Determination of mAb epitopes

Denatured vitronectin was coated on ELISA wells from 50 µl/well at concentrations between 1 and 4 µg/ml (13–52 nM) for 3 days at 4 °C. Unoccupied binding sites were blocked with 1% BSA in PBS. mAbs were used at concentrations between 0.25 and 1 µg/ml (1.7–6.7 nM) and pre-incubated with individual vitronectin peptides at 200 and 20 µg/ml (averaging 180 and 18 µM) in blocking buffer for 2 h. mAb/peptide mixtures were added to the vitronectin-coated wells and incubated on the plate shaker for a further 1.5 h. This was followed by peroxidase-conjugated rabbit anti-mouse IgG at a 1/1000 dilution for 1.5 h and ABTS for 10 min. Those peptides that displayed significant inhibition of mAb binding compared with controls were tested further at multiple dilutions to determine their 50% inhibition concentrations.

Binding of vitronectin to solid-phase mAbs

mAbs at 20 µg/ml in PBS, pH 7.1, were coated on to ELISA plates at 50 µl/well for 2–3 days at 4 °C to give a saturated coating. Following blocking with BSA in PBS, pH 7.1, and a rinse in PBS, 50 µl/well of 2-fold dilutions of plasma or denatured vitronectin in blocking buffer were added and incubated on a plate shaker for 2 h at room temperature. Following three 3 min washes with PBS, BiHV8 in blocking buffer was added to the wells coated with HV2 and BiHV2 was added to the remainder. Incubation was for 1.5 h on the plate shaker. After three more 3 min washes with PBS, streptavidin–peroxidase was incubated for 0.75 h at a dilution of 1/500 in blocking buffer. After four 3 min PBS washes, ABTS solution was added (100 µl/well) and colour development arrested after 3–30 min by addition of 25 µl of 6% oxalic acid. Absorbances were read on a Bio-Rad 3350 plate reader at a transmission wavelength of 405 nm, and a reference wavelength of 490 nm. In some experiments plasma or denatured vitronectin dilutions were pre-incubated with 13K or 6K heparin at 200 µg/ml for 1 h at room temperature before

adding the mixtures to the coated mAbs and continuing the ELISA as above.

Using this assay with mAb HV2 coated on the surface and BiHV8 as an indicator, the concentration of vitronectin in the plasma samples was determined by comparison of dilution binding curves with HV2-purified vitronectin, and was found to be between 200 and 400 µg/ml. In the plasma used in comparative ligand-binding experiments (see below) the concentration was close to 400 µg/ml.

Binding of heparin to mAb-tethered vitronectin

ELISA wells were coated with mAbs HV2 or HV11 as above. Vitronectin was bound from a dilution series of plasma or denatured vitronectin as described above. BiHep/BSA reagent (heparin conjugated to biotinylated BSA) was added at a 1/1000 dilution (approx. 0.5 µg/ml heparin, 38.5 nM) and incubated for 1.5 h on the plate shaker. In some instances unlabelled 13K or 6K heparin was included as a putative competitor of BiHep/BSA. BiHV8 was added to a parallel set of plates to monitor bound vitronectin concentration. Following incubation with biotinylated reagents, the remainder of the assay was as described above.

Controls

In all binding assays, negative controls were included, comprising omission of each component of the assay in turn, except for the enzyme conjugate and the substrate. For each assay that used biotinylated mAbs to measure the concentration of bound vitronectin, separate serial dilutions of plasma, purified native vitronectin and denatured vitronectin were coated to poly(vinyl chloride) wells, and the agreement between absorbances using BiHV2 and BiHV8 was checked. This was essential to ensure (i) the validity of quantitative comparisons and (ii) that comparisons of heparin binding were not confounded by differing concentrations of bound vitronectin under different conditions. In early experiments comparing the binding of purified denatured vitronectin to coated mAbs with that of native vitronectin from plasma to coated mAbs, plasma (from which the vitronectin had been removed by HV2-affinity columns) was added back to the denatured vitronectin sample. The presence of other plasma proteins did not affect the binding of denatured vitronectin to solid-phase antibodies and was omitted from subsequent experiments.

Statistical analysis

Multiple treatments were compared using ANOVA and Student–Newman–Keul's (SNK) test. Paired treatments were compared using Student's *t* test. Where the ratio, *R*, of mean ELISA absorbances M_1 and M_2 were calculated, the variance, *V*, of the ratio was calculated taking the two component absorbances as independent variables, using the following formula:

$$V = R^2[\text{var}(M_1)/M_1^2 + \text{var}(M_2)/M_2^2]$$

Where $\text{var}(M_1)$ and $\text{var}(M_2)$ are the variances of mean ELISA absorbances M_1 and M_2 respectively.

RESULTS

Binding characteristics of mAbs to native and denatured vitronectin

In a previous report [18] we described the binding of a panel of mAbs to coated purified human vitronectin, either native (pre-

Figure 1 Binding of vitronectin directly from plasma to coated mAbs compared with that of urea-denatured vitronectin (DVN)

mAbs were coated on ELISA wells at 20 $\mu\text{g}/\text{ml}$ in PBS for 48 h at 4 $^{\circ}\text{C}$. Plasma (A) and urea-denatured (B) vitronectin dilutions in blocking buffer (4–0.06 $\mu\text{g}/\text{ml}$; 53 nM–0.8 nM vitronectin), were incubated for 2 h at room temperature, followed by BiHV2 or BiHV8 for 1.5 h at room temperature. Bound biotin was detected with streptavidin-conjugated peroxidase and ABTS substrate. Data points are the means from duplicates in a typical experiment. The key for coated mAbs is shown in (B).

pared by affinity chromatography on mAb HV23), urea-denatured (prepared by affinity chromatography on heparin-Sepharose) or urea-denatured and reduced with 2-mercaptoethanol. With the exception of HV1, all the mAbs bound well to coated native vitronectin. Since the process of adsorption to plastic denatures native vitronectin to a variable degree [20], we re-addressed the binding characteristics of the mAb panel, using fresh plasma as a direct source of native vitronectin without any purification steps. Binding of native vitronectin from diluted plasma and urea-denatured purified vitronectin, from PBS to individual mAbs coated on ELISA wells, was detected using biotinylated mAbs which bound to different epitopes from the coated antibodies. The results in Figure 1(A) show that native vitronectin from plasma bound very poorly to coated HV1, in agreement with our previous findings for HV1 binding to coated native vitronectin. Although the remainder of the mAbs had all bound well to coated purified native vitronectin [18], when the mAbs were coated to the surface, widely different characteristics of vitronectin binding from plasma were observed (Figure 1A).

mAb HV3 bound plasma vitronectin poorly, with HV6 and HV16 also showing relatively weak binding. mAbs HV8, HV21 and HV23 showed considerable binding of plasma vitronectin, whereas HV2 and HV11 bound the highest amounts. In contrast to binding capacities for native vitronectin from plasma, the coated mAbs all showed similar capacities for binding urea-denatured vitronectin (Figure 1B), with the exception of HV1 whose binding of urea-denatured vitronectin was still not optimal, although much in excess of native plasma vitronectin.

Epitope mapping and choice of solid-phase antibodies for ligand-binding studies

The binding site of HV23 has been tentatively mapped to the C-terminal region of the somatomedin B domain of vitronectin ([18], and C. X. Moss, P. A. Underwood and B. Mabbutt, unpublished work). The binding sites of HV1, HV2 and HV11 were mapped using the set of peptides described in the Materials and methods section. HV1 binding to coated urea-denatured vitronectin was inhibited by a single peptide from the N-terminal region of the somatomedin B domain (AU16, amino acids 9–18) with 50% inhibition at 130 nM. HV2 binding was inhibited by a single peptide from the C-terminal end of the connecting region (AU42, amino acids 121–133) with 50% inhibition at 3.4 nM. HV11 was inhibited by peptides AU31 and AU32 from the N-terminal end of the connecting region (amino acids 75–84 and 85–94) with 50% inhibition at 4.9 nM and 230 μM respectively. HV11 was also inhibited by peptide AU20 from the haemopexin I domain (amino acids 175–184) with 50% inhibition at 320 μM . We were unable to identify epitopes for mAbs HV3, HV6, HV8, HV16 or HV21 using the panel of synthetic peptides. These mAbs bind poorly to vitronectin denatured with reducing agents [18] and probably bind to conformational epitopes. The placement of the binding sites of HV2 and HV11 within the connecting region of vitronectin fits with our previous findings for the characteristics of these two mAbs [18]. The connecting region, which is highly variable between species, is unlikely to have any biological ligand-binding function within vitronectin, as functionality is often linked with sequence conservation. Thus mAbs to this region would be good choices as solid-phase coating reagents to present vitronectin for ligand-binding studies. Coated HV2 and HV11 also displayed good binding of both plasma vitronectin and urea-denatured vitronectin. HV23 was included, as it binds native vitronectin from plasma relatively well and was used previously as an affinity purification reagent to prepare native vitronectin in our earlier studies [18]. For the present study HV2 was used as an affinity reagent for the purification of vitronectin from plasma. BiHV2 and BiHV8 were used to titrate vitronectin bound on the solid phase and BiHV1 was used to probe the degree of denaturation of bound vitronectin.

mAb HV2 denatures vitronectin

The degree of multimerization of vitronectin in plasma, vitronectin affinity-purified on mAb HV2 and urea-denatured vitronectin was compared by electrophoresis in native gels and Western blotting. This procedure detects all multimers, whether linked non-covalently or covalently. Figure 2 shows that negligible multimerization of vitronectin was detectable in plasma, whereas considerable multimerization had occurred with urea-denaturation of purified vitronectin, as expected. Considerable multimerization had also occurred following affinity purification from plasma on HV2, such that this material appeared similar to urea-denatured vitronectin. Affinity purification on HV2

Figure 2 Western blot showing detection of vitronectin monomer and multimer in plasma, affinity-purified vitronectin and urea-denatured vitronectin

We subjected 2 μ l of fresh plasma (Plasma), 2 μ g of urea-denatured vitronectin (DVN) or 4 μ g of vitronectin purified by affinity chromatography on HV2 (VN#7) to PAGE under native conditions, followed by Western blotting and detection by binding of mAb HV2. A different sample of fresh plasma (10 μ l; Plasma 2) was treated similarly on a separate occasion. The position of the vitronectin monomer is shown by the arrow.

involved two cycles of binding and elution, followed by passage over a Protein A–Sepharose column to remove a small proportion of HV2–vitronectin complexes that had leached from the HV2 column. This procedure gave much higher yields than the single-step purification on HV23 which we had used previously [18]. The vitronectin that eluted initially from HV2, however, existed in a partially pure state at high concentrations for relatively long periods between this first elution step from HV2 and final frozen storage, presenting an opportunity for multimer formation.

The question remained, was the vitronectin conformationally altered by binding to HV2, or did this occur only in solution after elution? Consequently we examined the conformational status of vitronectin bound directly from plasma to coated HV2, HV11 and HV23 by comparing the subsequent binding of BiHV1. This was done in parallel for plasma, HV2-purified native vitronectin and urea-denatured vitronectin. The results are shown in Figure 3. Binding of BiHV1 indicated denaturation of vitronectin, and the higher the absorbance, the greater the denaturation. Binding of BiHV8 was used to quantify the amount of vitronectin bound to the solid-phase mAb, from each of the three sources. It can be seen that similar amounts of vitronectin bound to coated HV2 from plasma, HV2-purified vitronectin or urea-denatured vitronectin. Binding of BiHV1 indicated a high degree of denaturation of urea-treated vitronectin and considerable denaturation of vitronectin purified by affinity chromatography on

Figure 3 Assessment of the degree of denaturation of different forms of vitronectin bound to solid-phase HV2 or HV11

mAbs HV2 or HV11 were coated on ELISA wells as for Figure 1. Plasma was incubated at 2% and HV2-purified and urea-denatured vitronectins at 8 μ g/ml (106 nM). BiHV1 was used to probe the conformation of bound vitronectin and BiHV8 to quantify bound vitronectin. Assay conditions were as for Figure 1. Data shown are means with range of duplicates from a representative experiment.

HV2, in agreement with the gels in Figure 2, although the degree of denaturation of the HV2-purified vitronectin, detected by BiHV1 binding, was not as great as that exhibited by urea-denatured vitronectin. Significant binding of BiHV1 was also observed to vitronectin bound directly from plasma on to coated HV2. This indicates that vitronectin bound to HV2 is held in a conformationally altered, partially denatured form. The results with coated HV11 were quite different. BiHV8 showed a slightly lower absorbance for plasma vitronectin compared with HV2-purified and urea-denatured vitronectin. The binding of BiHV1 to HV2-purified and urea-denatured vitronectins on coated HV11 was similar to that on coated HV2, reflecting their relative extents of denaturation. Binding of BiHV1 to vitronectin captured directly from plasma was, however, not significantly different from zero. This indicates that, unlike binding to coated HV2 which partially denatured the vitronectin, binding to HV11 maintained the native plasma conformation. The similar absorbances observed with BiHV1 on HV2-purified vitronectin bound to HV2 or HV11 suggested that a second round of binding of vitronectin to HV2 did not further denature it, and that further denaturation of vitronectin took place after elution of vitronectin from HV2 in the purification protocol. Coated HV23 gave similar results to coated HV11 and maintained native conformation (results not shown). The binding of BiHV1 to urea-denatured vitronectin presented on HV23, however, was considerably less compared with that observed with coated HV2 or HV11. Presumably the orientation of vitronectin bound to solid-phase HV23 was such that the approach of the second somatomedin B mAb, HV1, was sterically impaired. Consequently for heparin-binding studies, comparisons were restricted to vitronectin bound to the connecting-region mAbs, HV2 or HV11.

Native vitronectin bound to HV2 is denatured but not multimeric

To investigate whether native vitronectin bound to HV2 was multimeric as well as conformationally altered, its ability to bind BiHV2 and BiHV8 was compared. We reasoned that if the vitronectin bound to HV2 was multimeric, then BiHV2 would be

Figure 4 Test of multimerization of plasma vitronectin bound to mAb HV2

mAbs HV2 and HV11 were coated on ELISA wells as for Figure 1. Dilutions of plasma (A) or urea-denatured vitronectin (VN; B) were added in blocking buffer (range 4–0.06 $\mu\text{g/ml}$, 53–0.8 nM). Bound vitronectin was detected with BiHV2 or BiHV8. Assay conditions were as for Figure 1. Data points are the means from duplicates in a representative experiment.

able to bind to unoccupied epitopes in the multimer. Figure 4(A) shows that, although the binding of BiHV8 to plasma vitronectin held on HV2 is similar to that held on HV11, BiHV2 does not bind at all. This suggests that the plasma vitronectin bound to HV2 has not formed multimers. Figure 4(B) shows that when urea-denatured multimeric vitronectin is bound to HV2, there are still epitopes free in the multimers to bind BiHV2, thus verifying the method.

Heparin denatures plasma vitronectin

Plasma or urea-denatured vitronectin was pre-incubated with 13K heparin and then tested for its ability to bind to coated mAbs. Figure 5(A) shows that except for coated HV2, binding of plasma vitronectin to the coated mAbs was increased by pre-incubation with heparin. These differences were statistically significant ($P < 0.05$). Figure 5(B) shows that the binding of urea-denatured vitronectin to coated mAbs was not increased significantly by pre-incubation with heparin for any of them

Figure 5 Effect of pre-treatment of plasma or urea-denatured vitronectins with heparin on their ability to bind to coated mAbs

mAbs were coated on ELISA wells as for Figure 1. (A) Plasma at 0.25% in blocking buffer (13 nM vitronectin) was pre-incubated with 13K heparin at 200 $\mu\text{g/ml}$ (15 μM) for 1 h at room temperature before the mixture was incubated on the mAb-coated wells for 2 h. Bound vitronectin was detected with BiHV2 or BiHV8. (B) Urea-denatured vitronectin at 0.125 $\mu\text{g/ml}$ (1.7 nM) was similarly treated with heparin and added to coated mAbs. (C) 1% Plasma and urea-denatured vitronectin at 4 $\mu\text{g/ml}$ (53 nM) were pre-treated with heparin and subsequent binding to coated HV2 was detected with BiHV1 as a measure of denaturation. Assay conditions were as for Figure 1. Data shown are means $\pm$ range for duplicates (A and B) or means $\pm$ S.E.M. of quadruplicates (C) from representative experiments. * $P < 0.05$ or ** $P < 0.01$, significantly different from the respective paired control (Student's t test).

($P > 0.05$). Increased binding of vitronectin from heparin-treated plasma to the coated mAbs, which displayed a preference for urea-denatured vitronectin (see Figure 1), suggested that the

Figure 6 Effect of pre-treatment with heparins of different molecular masses upon binding of plasma vitronectin to coated mAbs

Coating conditions and heparin treatments were as for Figure 5. Plasma was used at 0.4% (21 nM vitronectin). Bound vitronectin was detected with BiHV2 (coated HV6 and HV21) and BiHV8 (coated HV2). Assay conditions were as for Figure 1. Data shown are means $\pm$ S.E.M. of quadruplicates from a representative experiment.

heparin was denaturing the vitronectin in the plasma. To test this hypothesis, the ability of BiHV1 to bind to vitronectin following heparin treatment was investigated. To avoid confounding the results of BiHV1 binding with quantitative differences in vitronectin binding (with or without heparin) to the coated mAbs, coated HV2 was used in this experiment. Thus we had observed that coated HV2 bound similar amounts of vitronectin from plasma before and after heparin treatment (see Figure 5A). Figure 5(C) shows that the vitronectin bound from heparin-treated plasma was more denatured than the control (i.e. higher binding of BiHV1). As we have shown that binding of vitronectin directly from plasma to HV2 already partially denatures it (see Figure 3), it is clear that pre-incubation with heparin has an additional denaturing effect. In contrast, pre-incubation of urea-denatured vitronectin with heparin had no significant effect on BiHV1 binding (Figure 5C), indicating that no further denaturation took place.

Does denaturation of vitronectin by heparin depend on multiple binding interactions?

The heparin used in the previous experiment had an average molecular mass of 13 kDa and can accommodate up to two molecules of vitronectin/molecule of heparin [13]. On the other hand, 6K heparin shows a stoichiometry of vitronectin binding of 1:1 [13]. We compared the effects of incubating these two forms of heparin with plasma on the ability of plasma vitronectin to bind to solid-phase mAbs in order to determine whether a similar degree of denaturation of vitronectin could be achieved at a stoichiometry of 1:1 heparin/vitronectin, as opposed to 1:2 heparin/vitronectin. The results are shown in Figure 6. Both 6K and 13K heparin treatments produced significant increases in binding of plasma vitronectin to coated mAbs HV6 and HV21 ($P < 0.01$; ANOVA and SNK test). There was, however, no significant difference between the two treatments. This indicates that denaturation of vitronectin by heparin does not require higher orders of binding than 1:1.

Figure 7 Comparison of the avidities of multivalent-heparin binding to plasma vitronectin or urea-denatured vitronectin presented on coated HV2 or HV11

mAbs HV2 and HV11 were coated on ELISA wells as for Figure 1. Dilutions of plasma or urea-denatured vitronectin were incubated for 2 h (range 2–0.125 μ g/ml vitronectin, 27–1.7 nM). Bound vitronectin was detected using BiHV8. Heparin binding was estimated using BiHep/BSA at 1 μ g/ml. The average molecular mass of BiHep/BSA was 133 kDa and approximately half the mass was heparin, giving 7.5 nM complex and 37.5 nM heparin. Assay conditions were as for Figure 1. On the abscissa of the graph 1 μ g/ml urea-denatured vitronectin also represents 0.25% plasma. For each biotinylated reagent the absorbance ratio was calculated for plasma vitronectin/denatured vitronectin. Data points are ratios and S.E.M. were calculated from the means of triplicate absorbance values from a representative experiment. Note that the values of the ratios for BiHV8 on coated HV11 were not significantly different from those on HV2 ($P > 0.05$; Student's *t* test for each point) and for the sake of clarity are not shown.

Is the avidity of the binding of heparin to vitronectin controlled by intrinsic affinity or multimeric interactions?

Binding of heparin to vitronectin held on surface-bound HV2 or HV11 was investigated by using BiHep/BSA reagent. This conjugate has approx. five heparin molecules/BSA molecule, thus making it large and multivalent compared with native heparin. In order to control for the confounding effect of amount of vitronectin bound to each coated mAb in each condition, BiHV8 was used to estimate bound vitronectin. The ratio of binding of BiHV8 to plasma vitronectin/urea-denatured vitronectin was calculated for each concentration of vitronectin added to coated mAb. This ratio was also calculated for corresponding binding of BiHep/BSA. These results are shown in Figure 7. Values less than 1 for the BiHV8 ratio indicated less plasma vitronectin than urea-denatured vitronectin bound to the coated mAb. Where the ratio of the BiHep/BSA was the same as the ratio of the BiHV8, this indicated that the amount of BiHep/BSA bound was directly proportional to the amount of vitronectin bound, and that the avidity of BiHep/BSA for plasma vitronectin was similar to that for urea-denatured vitronectin. This was observed at vitronectin concentrations of 2 μ g/ml (Figure 7) or above (results not shown). At these high input concentrations similar amounts of each form of vitronectin bound to the coated mAb (ratio not significantly different from 1), and similar amounts of BiHep/BSA bound to each form of vitronectin. At vitronectin concentrations below 2 μ g/ml there was increasing disparity between the BiHep/BSA binding ratio and the BiHV8 ratio, indicating a progressive decrease in binding avidity of the BiHep/BSA for the plasma form of vitronectin compared with the urea-denatured form. This indicated that at high vitronectin concentrations the bulky BiHep/BSA was able to bridge between individual plasma

Figure 8 Inhibition of binding of BiHep/BSA to plasma and denatured vitronectins by 6K and 13K forms of free heparin

mAbs HV2 and HV11 were coated on ELISA wells as for Figure 1. Plasma and urea-denatured vitronectin (DVN) were incubated for 2 h at 0.5% and 2 $\mu\text{g}/\text{ml}$ respectively (27 nM vitronectin). A series of dilutions of 13K (A) and 6K (B) heparin (starting at 750 $\mu\text{g}/\text{ml}$; 58 and 125 μM respectively) in blocking buffer were added to the wells in 50 μl volumes and incubated for 1 h at room temperature. BiHep/BSA at 3 $\mu\text{g}/\text{ml}$ (25 μl ; 22.5 nM complex, 113 nM heparin) was added per well without removing the inhibitors and incubated for a further 1.5 h. Final concentration of BiHep/BSA was 1 $\mu\text{g}/\text{ml}$ (7.5 nM complex, 37.5 nM heparin) and concentrations of inhibitor dilutions were 500, 100, 25, 6.25 and 1.56 $\mu\text{g}/\text{ml}$ (starting concentration 38.5 and 83 μM for 13K and 6K heparin respectively). Other assay conditions were as for Figure 1. Values are expressed as percentage absorbance in the absence of inhibitors. Data are means $\pm$ S.E.M. of triplicates from a representative experiment.

vitronectin molecules, thus effecting a multimeric interaction with an avidity approaching that for the already multimeric denatured vitronectin. As the concentration of the vitronectin decreased, spacing between individual vitronectin molecules would increase, making it increasingly difficult for the BiHep/BSA to bridge between them. Thus the binding of the BiHep/BSA was driven by the ability to form multimeric interactions, rather than intrinsic affinity, which would have remained constant over concentration differences. There was no detectable difference in the binding avidity of the BiHep/BSA to plasma vitronectin held on coated HV2 compared with HV11 in this experiment. This suggested that the conformational change of plasma vitronectin, which occurred upon binding to HV2, had little or no effect on the binding avidity of the BiHep/BSA.

To investigate in more detail the relative contribution of intrinsic affinity and multimeric interactions to the binding of

heparin to vitronectin, the ability of 6K and 13K heparin to inhibit the binding of BiHep/BSA was explored. The results are shown in Figure 8 as percentages of absorbance in the absence of inhibitors. 13K Heparin showed a very steep inhibition curve on urea-denatured vitronectin, its ability to cross-link component vitronectin molecules in the multimers making it a strong competitor with BiHep/BSA (Figure 8A). Similar inhibition curves were observed on urea-denatured vitronectin bound to coated HV2 or HV11, with 100% inhibition achieved on both at 25 $\mu\text{g}/\text{ml}$ heparin (1.9 μM). 13K Heparin was a weaker inhibitor of BiHep/BSA binding to plasma vitronectin than to urea-denatured vitronectin, due to reduced intrinsic affinity for plasma vitronectin, or reduced opportunity to form cross-links, or both. Inhibition was, however, significantly greater on plasma vitronectin bound to HV2 compared with HV11 ($P < 0.05$; ANOVA and SNK test). This indicates that the partially denatured vitronectin bound from plasma by HV2 has a higher intrinsic affinity for heparin than native plasma vitronectin held on HV11, since we know that this partially denatured vitronectin is not multimeric while still bound to HV2. This is borne out by the experiments of inhibition by 6K heparin (Figure 8B). In this case the inhibitory effects reflect the magnitude of the intrinsic affinity of the 6K heparin for individual vitronectin binding sites under all conditions, since this low-molecular-mass form cannot cross-link [13]. There are clear differences in affinity for 6K heparin between plasma vitronectin bound to HV11, plasma vitronectin bound to HV2 (on neither of which was 100% inhibition achieved over the concentration range tested) and urea-denatured vitronectin bound to either mAb. All three types of inhibition curve with 6K heparin were significantly less steep than their counterparts using 13K heparin ($P < 0.05$; Student's t test), which suggests a component of cross-linking by the latter, even with plasma vitronectin. The nature of the packing of the vitronectin monomers on the coated mAbs at the concentration of plasma used for this experiment may have brought monomers close enough for the 13K heparin to bridge on a random basis. This experiment, by using small free heparin molecules as competitors with the larger multivalent BiHep/BSA, enabled more sensitive detection of affinity differences between the different forms of vitronectin and demonstrated a clear hierarchy of binding affinity for heparin: urea-denatured multimeric > partially denatured monomeric vitronectin bound to HV2 > native vitronectin bound to HV11.

DISCUSSION

There are many reports in the literature of purification methods for vitronectin that reputedly yield the molecule in its native form [11,23–26]. We have identified a mAb, HV1, that does not show any binding activity to native vitronectin in plasma, when the mAb is coated on the solid phase. This mAb has a binding site in the somatomedin B domain of vitronectin, defined by a region of amino acid sequence difference between the human and the mouse molecule, between residues 9 and 18. It is exquisitely sensitive to changes in vitronectin conformation and demonstrates maximal binding to vitronectin that has been denatured fully with urea and reducing agents [18]. In this respect mAb HV1 has similar characteristics to the previously described mAb 153, whose binding site also lies within the somatomedin B domain [21]. We used mAb HV1 to probe the conformation of native vitronectin bound directly from plasma to various other mAbs with different epitope specificities. This revealed that vitronectin bound directly from plasma to mAbs HV11 and HV23 remained native, whereas that bound to mAb HV2 was conformationally altered/partially denatured. Furthermore, while still bound to HV2 the partially

Scheme 1 Putative interactions between different vitronectin conformers

Expansion of a scheme from Zhuang et al. [28,29]. Native vitronectin treated with strong dissociation agents unfolds progressively to produce denatured monomer. Upon removal of dissociating agent the denatured monomer refolds in physiological buffer down a different path to produce an unstable monomer, which multimerizes spontaneously. Interaction of native vitronectin with certain ligands (e.g. PAI-1) can directly produce this unstable form. Interaction of native vitronectin with other ligands (e.g. heparin, mAb HV2) produces a more stable partially unfolded form that converts to the unstable form at a slower rate. Refolding of the fully unfolded monomer in the presence of high salt rather than physiological buffer can direct correct refolding to the original form.

denatured vitronectin was not multimeric but formed multimers at some point following elution with MgCl_2 . Using the same system of solid-phase mAbs we demonstrated that incubation of plasma with heparin also partially denatured vitronectin, and that these two effects were additive, but still did not lead to fully denatured vitronectin. Increased binding of heparin-treated plasma vitronectin to denaturation-sensitive mAbs, using competition assays, has also been described by others [2,8,22]. Interaction of heparin with native vitronectin does not produce vitronectin multimers [2,8,22], even though the vitronectin is altered conformationally. This means that vitronectin purified by methods utilizing ligand binding (such as heparin–Sepharose or antibody affinity columns [11,23,24]) cannot be assumed to be native even though it appears to be monomeric. Preparations of plasma vitronectin most likely to remain native are those prepared by methods not involving ligand binding, such as those described by Dahlback and Podack [25] and Preissner et al. [26]. In addition, even if the preparation starts off in a monomeric form it can become progressively more denatured on storage ([4,14], and the present studies). Thus it is imperative in studies of vitronectin conformation to establish that the assumed native starting point is in fact truly native. We have solved this problem by binding native vitronectin directly from plasma to particular solid-phase antibodies which do not alter the native conformation, as determined by HV1 binding.

The partial denaturation of vitronectin by heparin and mAb HV2, while maintaining the monomeric form, appears to be similar in effect to that described for other mild denaturing conditions, such as 1 M urea and mild acidic conditions [2,11]. This is in marked contrast to treatments of plasma vitronectin with type 1 plasminogen activator inhibitor (PAI-1) or more highly sulphated glycosaminoglycans than heparin, such as

fucoidan, which result in rapid multimer formation [8,27]. Zhuang et al. [28] studied the unfolding of vitronectin in the presence of strong denaturing agents. Urea or guanidinium hydrochloride treatment of monomeric vitronectin produced unfolding curves indicating that the molecule progressively changed its conformation with increasing concentrations of denaturant but remained monomeric in the presence of the denaturant. Furthermore, refolding curves upon removal of denaturant showed hysteresis, demonstrating that the refolded molecule had a different conformation from the original monomer, and this new conformation had a very strong propensity to form multimers in physiological buffers [28]. This observation led the authors to propose a model of vitronectin conformation which included an unstable partially unfolded monomeric form that associated into multimers. Further studies showed that reversed folding of the chemically unfolded molecule down the original pathway to produce stable monomers indistinguishable from the original could be induced by refolding in high salt concentrations [29]. The overall conclusion from these studies was that there are two stable conformations of vitronectin under physiological conditions, native monomer or denatured multimer. We propose that binding of the stable native monomer to ligands, such as mAb HV2 and heparin, causes partial unfolding to a form that is relatively stable and converts slowly to the unstable form, which spontaneously multimerizes. PAI-1 on the other hand may convert the stable monomer directly to the unstable multimerizing form. Therefore, based on our findings and the literature reports to date, we propose an adaptation of the Zhuang et al. [28] model for interchange between different vitronectin conformers, shown in Scheme 1, which includes a partially denatured monomeric form that does not immediately form multimers.

There is still controversy over the ability of native vitronectin to bind heparin and the relative contributions of changed affinity compared with multivalency to the binding of multimeric vitronectin to heparin. Despite the elegance of recent fluid-phase binding studies of fluorescently labelled heparin to purified vitronectin, which either 'prove' [13] or 'disprove' [14] that native vitronectin can bind heparin, there is compelling evidence that vitronectin present in fresh plasma can bind heparin, resulting in alteration of the ligand-binding properties of the vitronectin ([6–8], and the present studies). Francois et al. [14] argued that reports of binding of plasma vitronectin to heparin were due to the presence of spontaneous dimers in the plasma vitronectin population. We have observed that platelet-poor plasma prepared from freshly collected blood contains no detectable high-molecular-mass forms, whereas outdated 'fresh-frozen plasma' from the blood bank (a common source of plasma for vitronectin preparation) does contain such forms. Consequently our experiments were performed on freshly prepared platelet-poor plasma that was frozen in small aliquots, stored for short periods of time and discarded after each use. Our experiments with binding of BiHep/BSA to vitronectin presented by mAbs on the solid phase indicated that multimeric interactions were the main contributors to binding, even with native plasma vitronectin, due to the large size (approx. 133 kDa) of the BiHep/BSA which could bridge relatively large distances. Experiments using 6K and 13K heparin as inhibitors of this reaction demonstrated a clear progression of binding affinity from the weakest on native plasma vitronectin presented on mAb HV11, to stronger affinity for partially denatured vitronectin presented on mAb HV2, to strongest affinity for urea-denatured vitronectin presented on either mAb. These results disagree with the claims of Zhuang et al. [13] who reported similar intrinsic heparin-binding affinities between native and multimeric vitronectin. In their studies, however, this similarity was only true if the binding reactions were done in the presence of a low salt concentration. At physiological salt concentrations the intrinsic affinity of heparin for native vitronectin approached an order of magnitude lower than that for urea-denatured vitronectin [13], which is not in disagreement with our findings.

Given that we can demonstrate differences in intrinsic affinities for heparin between native, partially denatured and urea-denatured vitronectin, the reason for these differences remains unknown. Recent studies by Francois et al. [14] of the binding kinetics of heparin to multimeric vitronectin have demonstrated a single fast association phase followed by a biphasic fast → slow dissociation phase, reflecting a weak initial binding interaction which is subsequently stabilized to a stronger bond. Weak heparin binding to native vitronectin may reflect a conformation which does not allow formation of the secondary stronger binding reaction. This may involve contribution of weak heparin-binding sites in the haemopexin I domain [30,31], although their contribution to heparin binding in the complete vitronectin molecule remains to be proven, and their orientation in the three-dimensional structure is unknown. Progressive refolding of the molecule towards the conformation present in the multimer may allow an increasingly permissive rearrangement to the more stable bond. This may or may not involve a progressive interaction of the two consensus heparin-binding sites present in the haemopexin II domain of vitronectin [8].

In conclusion, the available evidence to date (including the studies reported herein) indicates that there are heparin-binding regions of vitronectin exposed in the native molecule, occupancy of which alters its conformation. Our studies also indicate that the markedly increased binding of heparin by multimeric vitronectin is due to both increases in intrinsic affinity brought about

by changed conformation of the monomer, and by the availability of multivalent interactions between heparin and the vitronectin multimer.

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