Characterization of goat plasma vitronectin

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Received 28 November 2002; revised 10 January 2003; accepted 8 February 2003

Vitronectin (VN) was isolated and characterized from goat plasma in native and denatured state. Native VN consisted of 160 and >250 kDa polypeptides, whereas denatured VN showed bands of 81 and >250 kDa on SDS-gel. Storage of 81 kDa polypeptide for 3 days at 4°C resulted in formation of 160 and >250 kDa proteins. Hence high molecular weight forms of VN may be dimer and multimeric forms of 81 kDa monomer. Both native as well as denatured VN showed cell adhesive activity. Cells bound to native VN were round, whereas cells adhered to denatured VN were fully spread, a characteristic also observed with 81 kDa polypeptide. The 81 kDa VN bound to Heparin, whereas the 160 kDa preparation did not bind to Heparin in presence of urea. Absence of EDTA resulted in the degradation of goat VN. Similarly, addition of excess Ca²⁺ caused total degradation of VN polypeptides in buffers with EDTA, suggesting metalloprotease activity in the protein.

Key words: Goat, vitronectin

Vitronectin (VN) is a multifunctional plasma and extracellular matrix glycoprotein that participates in cell adhesion, blood coagulation, complement-dependent immunological pathways, differentiation of neuroepithelial cells into motor nerves and in the regulation of bone formation¹⁻⁴. It exists in at least two distinct forms in human plasma, the major one being a non-Heparin binding, 75 kDa monomer, while the minor form (about 2% of the plasma VN) has affinity for Heparin and is an aggregated species enriched in 65 kDa polypeptide⁵.

VN has not been characterized in detail from animal species and there is only one preliminary report on goat VN⁶. This protein has been implicated in the pathogenicity caused by facilitating bacterial adhesion to host cells⁷⁻¹¹. The present study, was undertaken to purify vitronectin from goat plasma and study its characteristics.

Materials and Methods

Materials

Goat blood was collected from animals maintained by the divisions of Pharmacology and Physiology of this institute with sodium citrate as an anticoagulant. Plasma was obtained by centrifugation of the blood at 10,000×g for 15 min at 4°C. To the plasma, PMSF was added to 1 mM final concentration. Heparin-Sepharose was purchased from Bangalore Genei, India and Sigma Chemical Co., USA, Sepharose 4 B from Pharmacia Fine Chemicals, Sweden, RGD peptide, PMSF and diaminobenzidine from Sigma Chemical Co., goat anti-rabbit IgG-HRP conjugate, protein molecular mass markers and nitrocellulose transfer membrane of Schleicher and Schuell were obtained from Bangalore Genei, India, Vero cells (African green monkey kidney fibroblasts) were generously provided by Dr Bhaskar Sharma of this division. All other reagents used were of the highest purity available.

Purification of native vitronectin by immunoaffinity chromatography

Immunoglobulin fraction from rabbit anti-VN 81 antiserum was fractionated by three cycles of ammonium sulfate precipitation (50% saturation) and coupled to CNBr-activated Sepharose 4B¹². Goat plasma (3-5 ml) was applied to immunoaffinity column (5 ml) pre-equilibrated with 20 mM Tris-HCl (pH 8.0)-0.5 M NaCl. Column was washed extensively with equilibration buffer, followed by elution of bound proteins with 0.2 M glycine-HCl (pH 2.2). The pH of the fractions was neutralized with 1 M Tris. This form of VN is referred as native VN.

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Abbreviations: VN, vitronectin; DTT, dithiothreitol; PMSF, phenyl methyl sulfonyl fluoride; DTNB, dithionitrobenzoic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.
Purification of denatured vitronectin by Heparin-Sepharose chromatography

This was performed as described before in presence of urea by the following steps: passage of plasma sequentially over Sepharose 4B and Heparin-Sepharose in presence of 20 mM sodium phosphate (pH 7.4)-100 mM NaCl-5 mM EDTA containing 0.05% sodium azide (PBS-EDTA). To the unbound plasma, urea was added to 8 M and kept at 20-25°C for 3 to 12 hr. This was applied to a 5 ml Heparin-Sepharose column equilibrated with 8 M urea in PBS-EDTA. After extensive washing, VN was eluted with 0.5 M NaCl. This form of VN is referred as denatured VN.

Isolation of 81 and 160 kDa vitronectin and generation of 27 and 53 kDa polypeptides

The 81 kDa polypeptide was isolated by reduction of denatured VN with 10 mM DTT followed by chromatography on Heparin-Sepharose. The buffer used was PBS-EDTA containing 8 M urea and 1 mM DTT. The bound 81 kDa polypeptide was eluted with 0.5 M NaCl. The 160 kDa VN was isolated from native VN fraction (see above) by dialysis against 20 mM sodium phosphate (pH 7.4), followed by DEAE-Sepharose chromatography. The 27 and 53 kDa polypeptides were obtained from purified 160 kDa VN by treating the protein with 4 M urea and 10 mM DTT. The polypeptides were separated by gel-filtration chromatography on Sephadex G-200 (2.5 × 50 cm column). The buffer used in chromatography was PBS containing 4 M urea and 5 mM DTT.

Cell adhesion assay

The assay was carried out by the method reported before. Microtitre plates were coated with 50 to 100 μl protein solution (10 or 20 μg/ml) and incubated overnight at 4°C. Wells were washed with PBS and free sites on plastic were blocked by adding 100 μl of 10 mg/ml heat denatured (70°C for 10 min) BSA. After 1 hr, wells were washed twice with PBS and 100 μl PBS with or without test material added followed by 35,000-50,000 vero cells in 100 μl RPMI 1640. Plates were incubated at 37°C for 60 min and unbound cells removed and wells washed gently with PBS. Adhered cells were fixed with 3% paraformaldehyde. For quantitation, fixed cells were stained with Coomassie blue, lysed with 2% SDS and absorbance read at 650 nm.

Iodoacetamide treatment, determination of sulfhydryl contents and assay for proteolytic activity

Iodoacetamide treatment of VN polypeptides was performed by reducing the polypeptides with DTT (10 mM final concentration), followed by incubating the reduced proteins with 60 mM iodoacetamide at 37°C for 5 hr. The treated proteins were dialyzed against PBS-EDTA and the sulfhydryl content determined using DTNB. The proteolytic activity of vitronectin was checked by including 25 mM CaCl2 with purified protein preparations and incubated at 37°C for 30 min. The control samples had no CaCl2. The degradation of polypeptides was visualized by SDS-PAGE and staining with Coomassie blue.

Electrophoresis, Western blotting and antisera production

SDS-PAGE was carried out either on 7.5% or 5-15% linear gradient gels. Native gel electrophoresis was performed essentially as SDS-PAGE, but the gel and running buffer did not contain SDS. Western blot was carried out, as before. In brief, protein bands were transferred from gel to nitrocellulose paper at 200 mA for 3 hr at 15°C, followed by blocking with 3% BSA. Rabbit anti-VN 81 antiserum was used at 1:200 dilution, whereas secondary antibody, goat anti-rabbit IgG-HRP conjugate, at 1:500 dilution. Enzyme reaction was measured by adding diaminobenzidine.

For antisera production, Heparin-Sepharose purified 81 kDa polypeptide was subjected to preparative SDS-PAGE, stained with Coomassie blue and destained. Stained 81 kDa polypeptide was cut, destained extensively with 50% methanol-10% acetic acid, followed by several washings with distilled water. Gel pieces were homogenized and extracted with PBS at 4°C overnight and the extract was used for antibody production. One rabbit was immunized with 50-100 μg of the extracted 81 kDa VN in Freund’s complete adjuvant and boosted every third week with the same amount of protein mixed with incomplete adjuvant. Blood was collected ten days after the third booster and presence of antibodies were confirmed by Western blot.

Results

Presence of high molecular weight vitronectin in goat plasma

Vitronectin isolated from freshly obtained blood by immunoaffinity chromatography showed three
prominent high molecular weight bands in SDS-PAGE (bands numbered as 1 to 3, Fig. 1A). Band 1 was present in the stacking gel, whereas band 2 barely entered the separating gel. These bands were of > 250 kDa. The prominent band 3 had an apparent size of 160 kDa. In addition, minor bands of 53 and 81 kDa were also observed. In initial experiments, the size of high molecular weight bands was determined using buffalo fibronectin (subunit size 230 kDa and 250 kDa) and human erythrocyte membrane proteins as marker proteins (not shown). Densitometry of stained gels revealed that bands 1 and 2 together constitute about 15-20%, whereas band 3 approximately 70% of the native VN protein. When SDS-PAGE of native VN was performed under reduced condition, bands of 27, 53, 68 and 83 kDa were observed with the disappearance of 160 and >250 kDa polypeptides. Further fractionation of native VN on DEAE-Sephose resolved into two major peaks: one that eluted with 100 mM NaCl and contained 160 kDa protein, which on reduced SDS-PAGE showed polypeptides of 27 and 53 kDa. The second peak eluting at 150 mM salt had >250 kDa polypeptides and this fraction after reduction showed bands of 68 and 83 kDa (Fig. 1A).

The results of immunoaffinity purification were confirmed by Western blot analysis of fresh goat plasma with rabbit anti-VN 81 antiserum. This experiment showed that about 50% of the VN in goat plasma was present as 160 and >250 kDa proteins and the rest was contributed by 81 kDa and other smaller polypeptides (Fig. 1B). The specificity of anti-VN antiserum was checked by its reaction with reduced human plasma in Western blot. A band of 65 kDa representing human VN was stained (Fig. 1C). In addition, a minor band of 48 kDa was also observed and may represent degradation product.

VN was also isolated in denatured state from goat plasma by Heparin-Sepharose chromatography in presence of urea. The denatured VN consisted of 81 kDa and two polypeptides of > 250 kDa. On reduction, it showed three major polypeptides of 53, 68 and 83 kDa (Fig. 1D). The slower mobility of 81 kDa polypeptide after reduction with an increase in size to 83 kDa suggests intrachain disulfide bonds in the protein.

Unfolding of 160 kDa vitronectin and generation of 27 and 53 kDa polypeptides

Initial attempts to separate 27 and 53 kDa polypeptides by gel filtration chromatography after treatment of 160 kDa VN with DTT were not

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**Fig. 1**—(A): SDS-PAGE of native VN isolated by immunoaffinity chromatography. [N, total native VN; 100 and 150 represent fractions of native VN eluted from DEAE-Sepharose with 100 and 150 mM NaCl, respectively. M, molecular weight markers]; (B): Western blot of goat plasma (0.2 μl) with rabbit anti-VN 81 antiserum. [Multimer (band 2), 160, 81, 68 and 53 kDa VN are marked by arrows]; (C): Reaction of reduced human plasma with rabbit anti-VN81 antiserum. [65 kDa human VN and minor 48 kDa band are shown by arrows]; (D): SDS-PAGE of denatured VN obtained after Heparin-Sepharose chromatography. [Multimers are marked by arrows]
successful, as both the polypeptides were observed in the same fractions after electrophoresis. However, these polypeptides were resolved by gel-filtration after denaturation and reduction of 160 kDa VN (Fig. 2A). These results suggest that unfolding of 160 kDa protein was essential before effective reduction of disulfides to generate 27 and 53 kDa chains. Reaction of DTNB with 160 kDa protein and the pattern of native gel electrophoresis corroborate this view. The 160 kDa protein did not react with DTNB; however, it reacted with DTNB in presence of urea (Table 1). Similarly, 160 kDa protein showed identical pattern with a diffused band in presence or absence of 2-mercaptoethanol in native gel electrophoresis (Fig. 2B).

**Formation of multimers from polypeptides**

Formation of multimers of 27, 53 and 81 kDa polypeptides were observed in in vitro. When denatured and reduced 27 or 53 kDa polypeptides were dialyzed against PBS or PBS-EDTA, high molecular weight bands were seen with the disappearance of parent protein. The mobility of these bands were similar to those of >250 kDa polypeptides observed in native and denatured VN preparations. Reduction of multimer formed from 27 kDa polypeptide resulted in reappearance of the parent protein and the formation of two additional minor polypeptides of 53 and 66 kDa. The reduction of multimers of 53 kDa polypeptide gave parent 53 kDa molecule and a new band of 66 kDa (Fig. 3A).

When denatured and reduced 81 kDa polypeptide were dialyzed against PBS-EDTA, no new high molecular weight band was observed in SDS-PAGE.
However, when dialyzed 81 kDa protein was stored at 4°C for 3 days, 160 kDa and higher molecular weight bands were observed. On further storage (7 days) at 4°C, no 160 kDa band was observed, but the intensity of bands in the stacking and at the top of the separating gels were enhanced. Incubation of 81 kDa protein at 37°C for 6-12 hr resulted in total disappearance of parent polypeptide and the appearance of bands in the stacking and at the top of the separating gels. Storage of 81 kDa VN in PBS-EDTA for 2 months at 4°C resulted in multimerization as well as degradation of the protein. The bands observed were 160 kDa and higher order multimers along with the degradation products of 53 and 68 kDa and lower size polypeptides (Fig. 3B). Absence of high molecular weight bands after treatment of reduced 27, 53 and 81 kDa polypeptides with iodoacetamide confirmed that the multimers were disulfide bonded (not shown).

**Binding of 81 kDa polypeptide to Heparin-Sepharose in presence of urea**

Binding of different VN polypeptides to Heparin-Sepharose was studied in buffers with or without urea. When plasma was passed over Heparin-Sepharose in PBS-EDTA, the fractions eluted from the column contained 53, 68, 81, 160 and >250 kDa polypeptides (Fig. 4A). However, in buffer with urea, only 81 and >250 kDa polypeptides of plasma bound to Heparin-Sepharose (Fig. 4B). Denatured VN in reduced state contained 53, 68 and 81 kDa polypeptides. Only 81 kDa polypeptide was retained when reduced denatured VN was passed over Heparin-Sepharose (Fig. 4C).
Cell adhesive characteristics of native and denatured vitronectin

Vero cells adhered to both native and denatured vitronectin, but the morphology of cells bound to these proteins was different. The cells bound to native VN were round, whereas they were fully spread on denatured VN. Also, the number of cells adhered to denatured VN was much larger (Fig. 5). To further characterize the polypeptide in denatured VN responsible for cell spreading activity, cell adhesion was performed with purified 27, 53, 81, 160 and >250 kDa polypeptides. All proteins supported cell attachment, but cell spreading was observed only with 81 kDa protein. Cell adhesion to native or denatured VN was inhibited by EDTA (90% inhibition at 5 mM) and Arg-Gly-Asp (RGD) peptide (80% inhibition at 200 μg/ml), but Heparin (up to 500 μg/ml) had no inhibitory effect. Cell adhesion was totally inhibited at 1:10 dilution of rabbit anti-VN 81 antiserum (not shown).

Proteolytic activity in vitronectin

Several lines of evidence suggest that goat vitronectin possesses proteolytic activity. First, degradation of 81 kDa polypeptide with the appearance of a new band of 53 kDa, was observed when denatured VN was dialyzed against PBS to remove urea. Inclusion of EDTA during dialysis prevented degradation of 81 kDa polypeptide suggesting that the proteolytic activity may be ion-dependent (Fig. 6A). This was confirmed when addition of Ca²⁺ to native and denatured VN resulted in total disappearance of all polypeptides present in these proteins. To pinpoint the polypeptide possessing proteolytic activity, Ca²⁺-induced degradation was tested with purified 27, 53, 81, 160 and >250 kDa polypeptides. As shown in Fig. 6B, these proteins were totally degraded in presence of Ca²⁺. The Ca²⁺-induced degradation was also observed with VN polypeptides recovered after electrophoresis or heat treatment at 100°C for 2 min (not shown).

Discussion

In this study, it was observed that a significant part of vitronectin in goat plasma exists as high molecular weight protein. Presence of aggregated form of VN in minor amount in human plasma⁵ and other species has been reported¹⁹. In an earlier study, denatured VN was shown to contain three polypeptides of 65 to 78 kDa

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Fig. 5—Attachment of vero cells to VN (A): 1, denatured VN; 2, 81 kDa VN and 3, BSA; (B): 1, native VN; 2, 160 kDa VN; 3, multimers (bands 1 and 2); 4 to 6 are 68 kDa, 53 kDa and 27 kDa VN
in reduced SDS-PAGE. The gel profile in absence of reducing agent was not reported. Our results on reduced denatured VN are in close agreement with this report.

The presence of a high molecular weight peptide and the appearance of low molecular weight polypeptides upon storage of 81 kDa VN suggest that this may be the monomeric form of the protein in goat blood. This is in agreement with the reported size of VN from other species. The 160 and >250 kDa proteins may represent dimer and multimers of 81 kDa polypeptide, respectively and the lower size bands may be the degradation products. The formation of multimer would require at least two free thiol groups per molecule. This is consistent with the presence of two free-SH groups in 81 kDa protein. Multimers of human VN were formed after denaturation followed by renaturation of the protein. However, multimers observed in native or denatured goat VN were not due to experimental artifacts, since multimers of similar sizes were observed in immunoblot of goat plasma.

The 81 kDa VN bound to Heparin-Sepharose both under physiological salt conditions and in presence of denaturant and reductant, suggest surface orientation of Heparin-binding site in native state. The 160 kDa VN did not bind to Heparin-Sepharose in presence of urea. Conformational changes during multimerization of human VN have been reported. The non-binding of 53 and 68 kDa fragments to Heparin in presence of urea may be due to the absence of Heparin-binding region, a situation analogous to the loss of Heparin-binding property observed in fragments generated from human VN after plasmin or thrombin digestion.

The cell adhesion to different VN polypeptides and the inhibition of cell attachment by RGD peptide suggest that this sequence is present and involved in VN-cell interaction. In human VN, this tripeptide sequence is the only site involved in cell adhesion.

Results presented here strongly suggest proteolytic activity in goat VN. Proteolytic activity in human VN has not been demonstrated fully. Earlier, formation of 65 and 10 kDa polypeptides from 75 kDa human VN was observed after prolonged storage of protein at 4°C or 30°C. It is unlikely that the proteolytic activity observed in goat VN was due to a contaminant for the following reasons. First, the activity was observed in different VN preparations i.e. >250, 160, 81, 68, 53 and 27 kDa polypeptides purified by different methodology. Secondly, the activity was also observed after denaturation with urea, SDS or heat treatment. This suggests that the proteolytic activity is independent of secondary or tertiary structure of the protein. Lastly, the multimers formed from 27 or 53 kDa chains on reduction gave newer bands in addition to parent polypeptide. The reduction may have exposed proteolytically susceptible/sensitive sites. The same mechanism was probably involved in the generation of 27 and 53 kDa chains from 160 kDa VN on reduction. It is important to note that the latent protease activity was observed in another extracellular matrix protein, fibronectin, that has both a Ca²⁺-dependent serine protease and a collagenase activity in the molecule. The significance of proteolytic activity in goat VN is not clear. This activity in vivo may generate functional fragments or may be involved in remodelling the extracellular matrix.

Acknowledgement
Financial assistance from ICAR, New Delhi and IVRI, Izatnagar (UP) to SS, VA and TG is...
duly acknowledged.

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