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VITRONECTIN IS A SUBSTRATE FOR TRANSGLUTAMINASES+

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Summary: Vitronectin (VN) was found to be a substrate for both plasma transglutaminase (Factor XIIIa) and guinea pig liver transglutaminase (TG). Incorporation of [^{3}H]-putrescine indicated the presence of reactive glutaminyl residues in VN. When VN was incubated with TG or Factor XIIIa, in the absence of putrescine, multimeric covalent complexes were identified, indicating that VN can also contribute lysyl residues to the bond catalyzed by transglutaminases. Cross-linking of VN by TG and Factor XIIIa may modulate the effects of VN on the complement and coagulation systems in hemostatic plugs and extracellular matrix.

Introduction: Vitronectin (VN) or "serum spreading factor" is an adhesive glycoprotein which promotes cell attachment and spreading (1-3). Despite the functional similarity with fibronectin, and the common Arg-Gly-Asp cell recognition sequence, the two proteins share virtually no sequence homology (4). VN is identical however to S-protein, the inhibitor of the membrane attack complex of complement (4,5). VN may also have a regulatory role in the clotting system by retarding the inactivation of thrombin by antithrombin III (6,7), by neutralizing heparin and heparan sulfate (8), and by binding to stimulated platelets (9).

VN is present in plasma at 200-400 μg/ml and has also been detected in the extracellular matrix of tissues, including atherosclerotic plaques and carcinomas (10-13). VN has a binding site for collagen (14), a prominent component of extracellular matrix.

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Abbreviations: VN: vitronectin; TG: guinea pig liver transglutaminase; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA: ethylenediamine tetracetic acid.

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Recently, Factor XIIIa has been demonstrated to mediate incorporation of fibronectin into extracellular matrix (15). Because of the functional simlarities between fibronectin and vitronectin, we investigated whether VN might also be a substrate for Factor XIIIa. In addition, VN can be detected in endothelial cells (16) and promotes their attachment and spreading (17). Our lab has recently identified a transglutaminase in endothelial cells which cross- reacts immunologically with guinea pig liver transglutaminase (TG) and which is active in crosslinking and stabilizing fibrinogen (18). We therefore investigated whether TG might also utilize VN as a substrate.

Materials and Methods: All chemicals were of reagent grade. 2,3-[^{3}H] putrescine dihydrochloride (38.7 Ci/mmol) and 1,4-[^{14}C] putrescine dihydrochloride (104.6 mCi/mmol) were obtained from New England Nuclear. Guinea pig liver transglutaminase (19), human platelet Factor XIII (20), and vitronectin (21) were purifed as previously described. The platelet Factor XIII (150 μg) was activated to form Factor XIIIa by incubation with α-thrombin (100 units/ml) for 30 minutes at 37^{0}C. Thrombin was inhibited by addition of D-phenylalanyl-L-prolyl-L-arginine chloromethylketone (PPACK) at 200 μM. Appropriate dilutions of Factor XIIIa were then used in assays.

Incorporation of [^{3}H]-Putrescine into VN: The incorporation of [^{3}H]-putrescine by Factor XIIIa or TG into VN or casein was performed as previously described (22). The reaction was carried out in 0.1 ml of buffer containing 50 mM Tris-HCl (pH 8.5), 20% (v/v) glycerol, 250 μM putrescine, 1 μCi [^{3}H] putrescine, 20 mM dithiothreitol, 2.5 mM calcium chloride, either Factor XIIIa (15 μg/ml) or TG (25 μg/ml), and the substrate to be assayed. Substrate concentrations used were VN 0.2 mg/ml and casein 1 mg/ml. Reaction mixtures were incubated for 30 minutes to 20 hours at 37 ° C and the reactions stopped by addition of 0.1 ml of 50% trichloroacetic acid. The precipitate was collected on Whatman GF/C filters, washed 3 times with 10 ml of 5% trichloroacetic acid, and the radioactivity measured by liquid scintillation.

[^{14}C]-Putrescine incorporation into VN and SDS-PAGE: This assay was performed as described for [^{3}H]-putrescine incorporation except that 0.001 μCi of [^{14}C]-putrescine per reaction was used and VN concentration was changed to 40 μg/ml. Reactions were stopped at various times with reducing buffer containing 1% sodium dodecyl sulfate, 1.5 M urea, 2.5 mM EDTA, 30 mM Tris-HCl (pH 7.5), 5% 2-mercaptoethanol, and 0.001% bromophenol blue, and then boiled for 3 minutes. The proteins were then separated on 4-15% or 6-15% linear gradient polyacrylamide gels with 3% stacking gels (23). Gels were then stained in Coomassie Brilliant Blue followed by destaining and fluorography (24). Molecular weights were determined by comparison with a [^{14}C] methylated protein mixutre from Amersham.

Immunoblotting: was performed as previously described (19). A 1:1000 dilution of murine monoclonal antibody to VN (Cytotech, San Diego, CA) was incubated in Tris-buffered saline (150 mM NaCl, 10 mM Tris, pH 7.4) containing 1% bovine serum albumin for 16 hours followed by a 1:1000 dilution of goat anti-mouse horseradish peroxidase conjugate (BioRad, Richmond, CA) for 4-6 hours. The nitrocellulose was developed by a color reagent from BioRad. Molecular weights were estimated by comparison with both prestained (range: 17,000-130,000) and unstained (range: 42,699-200,000) molecular weight standards from BioRad.

Silver Staining: of gels was performed after SDS-PAGE using a kit available from Sigma.

Results: The time course of [^{3}H] putrescine incorporation into VN by TG (25 μg/ml) and Factor XIIIa (15 μg/ml) are shown in Figure 1. Under identical conditions, these concentrations of enzymes gave equivalent [^{3}H]-putrescine incorporation into casein. The incorporation by TG proceeded at approximately ten times the rate of Factor XIIIa, and the

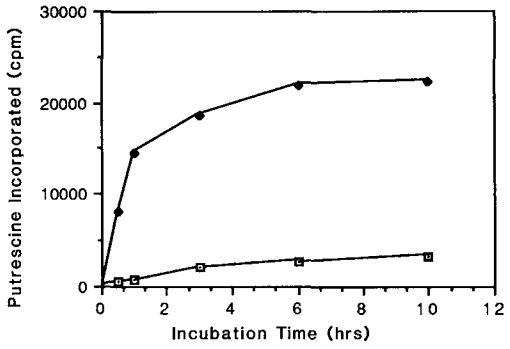

FIGURE 1: INCORPORATION OF [^{3}H]-PUTRESCINE INTO VN BY TG AND FACTOR XIIIa.

[^{3}H]-Putrescine (78,400 cpm per nanomole) was incubated with VN (0.2 mg/ml) and either TG (◆) or Factor XIIIa (□). The reaction was stopped at the indicated time intervals with trichloracetic acid and incorporated counts measured by liquid scintillation.

difference was maintained even at 20 hours incubation (data not shown). At the end points of the incubation time courses, TG had incorporated 0.9 mole putrescine per mole of VN while only 0.1 mole putrescine/mole VN was incorporated by Factor XIIIa. However, it is difficult to determine the number of reactive glutaminyl residues with certainty (25) since VN is crosslinked to itself (shown below). Since VN could compete with [^{3}H] putrescine for incorporation, a serious underestimation of reactive glutaminyl residues would result.

The incorporation of [^{14}C]-putrescine by TG and Factor XIIIa was examined by SDS-PAGE. No [^{14}C]-putrescine incorporation occurred in the presence of EDTA (10 mM) or with a 100-fold molar excess of unlabeled putrescine (data not shown). In the presence of 2.5 mM calcium chloride, both bands of VN were heavily labeled after 10 minutes (Figure 2A and 2 B, lane l). At 30 and 60 minutes, high molecular weight multimers (up to 6-mers) of VN were visible with TG (Figure 2A, lanes 2 and 3). At one hour incubation, Factor XIIIa had catalyzed formation of a VN-dimer but no higher molecular weight multimers were visible (Figure 2B, lane 3). This dimer was composed of 3 distinct bands of approximate molecular weights 149 kDa, 126 kDa and 138 kDa, probably corresponding to homodimers of the 74 kDa and 63 kDa bands, and a heterodimer of each, respectively. The molecular form of the VN-dimer catalyzed by TG was further examined by silver staining of a 6-15% SDS-PAGE gel (not shown), confirm - ing the triplet composition of the VN-dimer.

The patterns of [^{14}C]-putrescine incorporation into VN catalyzed by Factor XIIIa and TG were distinct. Factor XIIIa incorporated [^{14}C]-putrescine equally into the 74 kDa and 63 kDA bands, while TG-induced incorporation occurred preferentially the 74 kDa VN form (compare Figures 2A and 2B, lanes l). In addition, a new band of approximate molecular weight 54 kDa was visualized during TG-but not Factor XIIIa- induced incorporation. The origin of this band is currently unknown. Differences in substrate

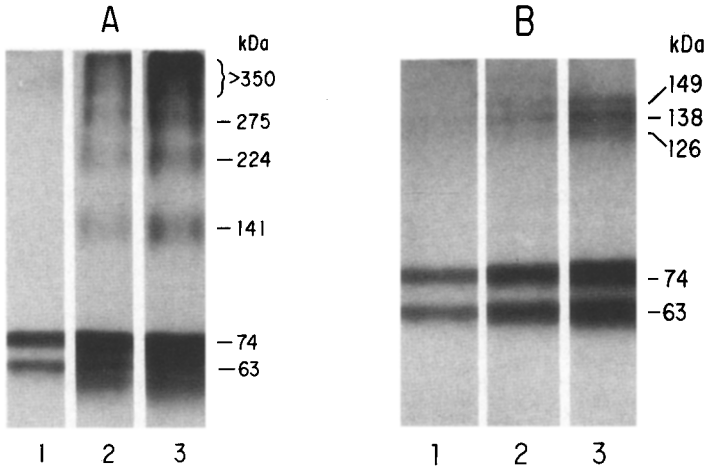

FIGURE 2: TIME COURSE OF [^{14}C]-PUTRESCINE INCORPORATION INTO VN BY TG (2A) AND FACTOR XIIIa (2B): ANALYSIS BY SDS-PAGE AND FLUOROGRAPHY.

VN (40μg/ml) was incubated in the presence of [^{14}C]-putrescine and TG (2A) or Factor XIIIa (2B). Incubation times were 10 minutes (lanes 1), 30 minutes (lanes 2), and 60 minutes (lanes 3). The reactions were stopped by adding reducing buffer followed by SDS-PAGE on 4-15% linear gradient gels and fluorography. Exposure times were 40 hours (2A) and 120 hours (2B).

specificities between Factor XIIIa and TG, due chiefly to the broader substrate specificity of the latter enzyme, have been previously noted (26).

Since putrescine is a bifunctional primary amine and could possibly ligate two VN molecules, the cross-linking of VN by TG and Factor XIIIa in the absence of putrescine was repeated using SDS-PAGE and immunoblotting. As seen in Figure 3, identical results were obtained, indicating that the high molecular weight bands were indeed due to VN-VN crosslinks. Similar results were obtained when VN was incubated with TG (data not shown).

Discussion

The data presented demonstrate for the first time that vitronectin is a substrate for both plasma and tissue transglutaminases. Incorporation of [^{3}H]-putrescine demonstrates the presence of reactive glutaminyl residues, while the formation of VN multimers indicates that there are also lysyl residues in VN capable of forming the ε-(γ-glutamyl) lysyl amide bonds catalyzed by transglutaminases. Studies are currently underway in our lab to define the identity of the glutamines and lysines in VN (4) involved in cross-linking. Although VN will spontaneously form disulfide-linked dimers (21), such a mechanism could not explain the multimeric VN complexes we observed, since SDS-PAGE was performed under reducing conditions, and since complex formation was dependent on the presence of enzyme and calcium ions.

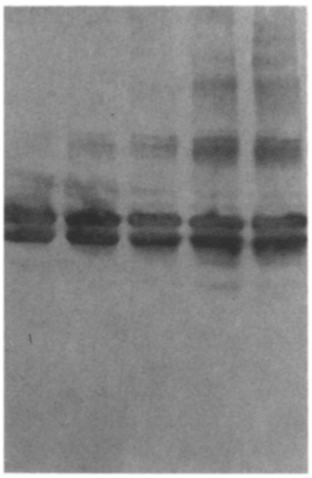

FIGURE 3: IMMUNOBLOT OF VN CROSS-LINKING BY FACTOR XIIIa.

VN (100μg/ml) was incubated with Factor XIIIa (15μg/ml) for various time intervals. The reactions were stopped at 10 minutes (lane 1), 20 minutes (lane2), 30 minutes (lane 3), 45 minutes (lane 4), and 1 hour (lane 5) by reducing buffer, followed by SDS-PAGE with 4-15% linear gradient gel and immunoblotting.

Although Conlan et al (10) did not detect covalent complexes in samples of sera after clotting platelet-poor plasma, the Factor XIIIa-mediated cross-linking may require higher Factor XIIIa concentrations as provided by platelets (27). Alternatively, the reaction may occur primarily at the surfaces of cells with VN receptors, (eg. platelets (9) and endothelial cells (28)), or preferentially in the extracellular matrix. The nine-fold greater putrescine incorporation with TG versus Factor XIIIa may indicate that VN cross-linking occurs primarily in tissues. In addition to the noncovalent interactions with glycosaminoglycans and collagen, transglutaminases may provide another mechanism for interactions of VN with matrix components by catalyzing covalent complexes with other matrix substances which are also transglutaminase substrates (fibronectin, collagen, thrombospondin).

The formation of cross-linked VN multimers may be accelerated or inhibited by molecules which bind VN (heparin, collagen, antithrombin III, thrombin). Whether the VN multimers are functionally active in cell spreading, inhibition of membrane attack complex formation, or regulating coagulation is under investigation.

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