Published in final edited form as:
J Trauma. 2011 August ; 71(2 Suppl 1): S171–S175. doi:10.1097/TA.0b013e31822556f4.

Poly-*N*-Acetylglucosamine Fibers Amplify the Effectiveness of Recombinant Factor VIIA on Clot Formation in Hemophilia B Canine Blood

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Abstract

Background—Achieving hemostasis in anticoagulated patients is an increasingly important clinical issue. Poly-*N*-acetylglucosamine (pGlcNAc) nanofibers activate platelets by β_3 subunit (CD61) and the von Willebrand receptor GP1b (CD42b) integrin signaling for generation of a prothrombotic surface membrane. Recombinant coagulation factor VIIa (rFVIIa) functions in hemophilia A and B by catalyzing formation of the Xa/Va complex on the surface of activated platelets. These observations suggest that pGlcNAc nanofibers may amplify the activity of rFVIIa in hemophilic blood.

Methods—The activity of rFVIIa on platelets was tested by performing thromboelastographic analysis with blood from hemophilia B dogs in the presence of pGlcNAc nanofibers and increasing concentrations of rFVIIa. Mechanisms for hemostatic system activation were investigated with inhibitors of tissue factor, factor XIIa, and platelet function.

Results—Recombinant FVIIa was observed to partially restore the ability of the hemophiliac blood to form fibrin clots in a dose-dependent manner with thromboelastographic analysis. The addition of pGlcNAc nanofibers amplified the rFVIIa effect. The activity of rFVIIa and the amplification effect of pGlcNAc were dependent on platelet integrin function but independent of FXIIa and tissue factor activities.

Conclusions—The pGlcNAc nanofibers amplify rFVIIa activity in hemophilia B canine blood by activating platelets through integrin-dependent mechanisms.

Keywords

Factor VIIa; Poly-*N*-acetyl glucosamine nanofibers; Hemophilia B; Thromboelastogram; Corn trypsin inhibitor

The control of hemorrhage is an important clinical issue in hemophilia patients and in patients who are treated with anticoagulation and antiplatelet drugs. Achieving surface hemostasis in these patients often presents critical challenges in treatment during surgery and traumatic injury. Two clinical approaches have been advanced in the last decade that aid in achieving hemorrhage control in patients with nonsurgical bleeding disorders: recombinant coagulation factor VIIa (rFVIIa, NovoSeven) as an agent for the treatment of

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hemophilia A and B patients[1,2] and poly-*N*-acetylglucosamine nanofibers (pGlcNAc nanofibers, formulated as the Syvek patch or mRDH hemostat) for hemorrhage control in anticoagulated patients.[3,4] Both rFVIIa and pGlcNAc interact with platelets to produce hemostasis.

The mechanisms for rFVIIa-mediated hemostasis in hemophilia A and B are thought to involve an rFVIIa interaction with the platelet surface for the direct activation of platelet-associated factor X,[5,6] without the need of factors IX and VIII. This hypothesis has been supported by clinical findings that rFVIIa can improve hemostasis in patients with defective coagulation,[7] thrombocytopenia,[8-10] or thromboasthenia,[11,12] and patients subjected to trauma with bleeding disorders.[13,14] However, high doses of rFVIIa are frequently required for hemostasis, because of the low affinity of this coagulation factor for the platelet surface. The presence of activated platelets at the site of injury stimulates the coagulation cascade leading to thrombin generation.

The pGlcNAc nanofibers have proven effective in generating hemostasis through mechanisms that include platelet activation.[15-18] Nanofibers of pGlcNAc have been formulated into products for accelerating hemostasis at femoral access sites in cardiac catheterization procedures (Syvek patch)[3] and for controlling massive hemorrhage in liver injury (mRDH).[4] Nanofiber pGlcNAc promotes hemostasis at wound sites by inducing red blood cell (RBC) agglutination[19] and endothelial-dependent vasoconstriction.[20] The contact of platelets with the polysaccharide nanofiber results in a platelet activation response that includes shape change, platelet p-selectin expression, phosphatidylserine (PS) surface exposure, and the activation of platelet β_3 subunit (CD61) and the von Willebrand receptor GP1b (CD42b) integrins.[17]

A “non-classical” mechanism has been recently identified for pGlcNAc nanofiber activation of hemostatic systems. RBCs tightly bind to pGlcNAc nanofibers rapidly on contact with blood.[19] The result of this interaction is an alteration in band 3 to produce a stomatocytic morphology by the RBCs. A result of this shape change is surface PS exposure and the activation of the intrinsic coagulation cascade to generate thrombin on the surface of the RBCs.[21,22] The prothrombotic state of pGlcNAc-bound RBCs is similar to that observed in several pathologic conditions, including uremia,[23] sickle cell disease,[24] and β-thalassemia,[25] where the surface exposure of PS and subsequent activation of the coagulation cascade was demonstrated.[26] The prothrombotic interaction of pGlcNAc with RBCs has been shown to be a specific property of the tertiary structure of the pGlcNAc chains in a highly crystalline parallel β-conformer state.[16]

The ability of pGlcNAc to activate platelets along with the known rFVIIa bypass activity indicated above suggests the hypothesis that pGlcNAc nanofibers might amplify the rFVIIa bypass effect at topical hemorrhage sites. This hypothesis is supported by previous findings that pGlcNAc provides surface hemostasis when applied to bleeding sites produced in the spleen of hemophilia B dogs.[27,28] In this article, the kinetics of fibrin gel clot formation in hemophilia B canine blood was assessed as a function of pGlcNAc and rFVIIa.

MATERIALS AND METHODS

Materials

Recombinant FVIIa (NovoSeven) was purchased from NovoNordisk A/G, and pGlcNAc nanofiber preparations were provided by Marine Polymer Technologies (Danvers, MA). Canine hemophilia B blood, from the Chapel Hill colony at the Francis Owen Blood Research Laboratory, was freshly drawn into citrated anticoagulant and used for the experiments. Antitissue factor antibody (clone HTF-1) was purchased from Beckton-

Dickson Biosciences. Integrilin (eptifibatide) was obtained from COR Therapeutics (San Francisco, CA), and Corn Trypsin Inhibitor was purchased from Hematological Technologies Nitrosoglutathione was a gift from Dr. Mark Sheonfisch (Department of Chemistry, University of North Carolina at Chapel Hill). Dade thromboplastin C was obtained from Baxter Diagnostics (Deerfield, IL), whereas chitosan was purchased from Sigma-Aldrich (St. Louis, MO).

Blood Collection and Processing

Peripheral blood was obtained from FIX-deficient dogs at the Francis Owen Blood Research Laboratory at the University of North Carolina at Chapel Hill. This line of hemophilia B animals lack expression of the FIX gene.[23] A volume of 4.25 mL of blood was drawn into a 5-mL syringe that contained 0.75 mL of 3.2% (wt/vol) citrate with pH 7.4. Platelet-rich plasma was obtained by centrifuging the blood at 300 *g* for 10 minutes at room temperature.

Thromboelastogram Data Collection and Analysis

Thromboelastographic (TEG) measurements were performed with a TEG-5000 Thrombelastograph Hemostasis Analyzer (Hemoscope Corporation, Niles, IL). Assays were initiated by adding 10 mmol/L $CaCl_2$ to citrated whole blood and then immediately transferring 327 μL of the calcified blood to the TEG chamber that contained 33 μL citrated saline (negative control) or citrated saline with 350 μg pGlcNAc nanofibers or chitosan. In selected experiments, pharmacological agents were added to the blood 15 minutes before initiation of the assay, and rFVIIa was added (100× concentrated) immediately before adding calcium. Measurements were repeated as indicated in the Figures (n value). Relevant parameters (the time for initiation of the clot, *R*, the time in minutes; θ, the slope of the curve during the polymerization of the clot; and MA, the maximum amplitude of the clot) together with the average deviation values were reported. *p* values were calculated using the Student's *t* test.

RESULTS

TEG analysis showed that rFVIIa partially restored the ability of canine hemophilia B blood to clot in a dose-dependent manner (Fig. 1). However, even the highest rFVIIa dose of 100 nM did not produce normal fibrin clot formation. A comparison of the upper and lower panels of Figure 1, with and without pGlcNAc, show that the inclusion of pGlcNAc nanofibers in the TEG reaction mixture significantly amplified rFVIIa acceleration of fibrin polymerization.[29] The nanofibers decreased the time for initiation of clot formation (*R* value), increased the rate at which clot formation occurred (θ value), and produced a stiffer clot (MA value). The differences between *R*, θ, and MA values with and without pGlcNAc were statistically significant ($p < 0.05$) at every rFVIIa concentration. Even in the absence of rFVIIa, pGlcNAc was able to initiate fibrin polymerization (upper right panel, Fig. 1).

Figure 2 shows TEG data with platelet inhibitors, and with or without pGlcNAc, in the presence of 30 nM rFVIIa. The results in Figure 2 show that the integrin inhibitor Integrilin (eptifibatide) at 50 μmol/L diminished the rate of fibrin polymerization and the final clot stiffness. Inhibition of overall platelet function with the cAMP agonist Iloprost at 10 μmol/L and the NO donor nitrosoglutathione at 100 μmol/L to stimulate platelet cGMP had a similar effect on fibrin polymerization.

The data in Figure 3 show that tissue factor inhibition did not affect fibrin polymerization with rFVIIa in the presence or absence of pGlcNAc. The antitissue factor antibody inhibited tissue factor acceleration of rFVIIa activity in the TEG system. The addition of a tissue factor source to the hemophilia B blood along with rFVIIa accelerated both initiation of

fibrin polymerization and the rate of fibrin clot formation; this acceleration effect was inhibited by the antibody (data not shown). Corn trypsin inhibitor that inhibits factor XIIa and the intrinsic coagulation pathway did not affect fibrin clot formation in the presence of pGlcNAc and/or rFVIIa.[17,30]

The TEG curves in Figure 4 were obtained in the presence of rFVIIa with or without chitosan, another polysaccharide material used in hemostatic bandages.[31] In contrast to the potentiating effect of pGlcNAc on rFVIIa activity, chitosan did not accelerate the initiation of fibrin clot formation as reflected by the *R* values, and slowed in a small, but statistically significant manner, the rate of polymerization as indicated by the decrease in the θ parameter and generation of clot stiffness as reflected in the lower MA value with chitosan. In addition, chitosan alone did not initiate clot formation as did pGlcNAc nanofibers.

Figure 5 shows that in both whole blood and plateletrich plasma, rFVIIa with pGlcNAc nanofibers caused clot formation, whereas rFVIIa alone showed diminished clot formation in whole blood and no clot formation in plateletrich plasma. The effect of rFVIIa and pGlcNAc-rFVIIa mixtures on the rate of fibrin polymerization was clearly more pronounced in whole blood than in platelet-rich plasma.

CONCLUSIONS

The results in this article show that pGlcNAc nanofiber activation of platelets leads to an amplification of rFVIIa activity in hemophilia B dog blood. The *N*-acetyl-polyglucosamine nanofibers accelerated the kinetics of fibrin polymerization associated with a broad range of rFVIIa concentrations through mechanisms that are sensitive to platelet inhibition. The importance of platelets in pGlcNAc amplification of rFVIIa is demonstrated by the sensitivity of pGlcNAc and rFVIIa effects to inhibit integrins, either specific inhibition by Integrilin or nonspecific inhibition by platelet cyclic nucleotide agonists cAMP and cGMP. The observation that platelet integrin inhibition largely eliminates the accelerating effects of pGlcNAc and rFVIIa on clot formation is consistent with the previous finding that integrin signaling is a dominant mechanism through which pGlcNAc nanofibers active platelets.[17] The ability of pGlcNAc to interact with rFVIIa is dependent on the specific molecular structure of the nanofiber. Chitosan,[31] a polysaccharide with an amorphous structure, did not accelerate thrombin activation and adversely affected the structural integrity of the fibrin clot. This result is consistent with and related to the previous finding that pGlcNAc, but not chitosan, activates platelets and factor XII[17,27,30] and strongly interacts with RBCs to produce stomatocytes and generate thrombin.[21,30]

The data indicate that the platelet-pGlcNAc interaction is of fundamental importance in the rFVIIa combination experiments and that interaction leads to hemostasis. The observation that the fibrin polymerization kinetics is faster in whole blood than platelet-rich plasma suggests that RBC-dependent mechanisms are also important. The RBCs and platelets interact in vivo to stimulate platelet thromboxane synthesis and RBCs' exposure of PS like platelets generates thrombin. Several pathologic conditions involving RBCs, including uremia,[23] sickle cell disease,[24] and β-thalassemia,[25] are associated with the surface exposure of PS and subsequent activation of the coagulation cascade (see ref. 26 for a review). The data suggest that the interaction of RBCs with pGlcNAc fibers also leads to PS exposure on the surface of the RBC leading to the generation of thrombin to produce hemostasis.[21,22]

These observations that the pGlcNAc nanofiber material can amplify the activity of rFVIIa in hemophilia B canine blood suggests that type A and B hemophiliacs might benefit from the use of pGlcNAc nanofiber-based products to control bleeding in surgery and from traumatic injury. Materials such as pGlcNAc nanofibers may interact with rFVIIa under

other conditions where coagulation factors are present but at reduced levels and/or are dysfunctional. For example, in trauma-induced coagulopathies with reduced FIX levels, rFVIIa has been shown to activate FIX in a reaction that is dependent on platelet activation.[32] The activation of platelets by contact with pGlcNAc nanofibers potentiates the activation of FIX by rFVIIa. Further studies are required to further explore these observations.

Acknowledgments

We thank the technical staff at the Francis Owen Blood Research Laboratory for their help in maintaining the hemophilia dogs that this research depended on.

Supported by funds from Marine Polymer Technologies, Inc., Danvers, MA.

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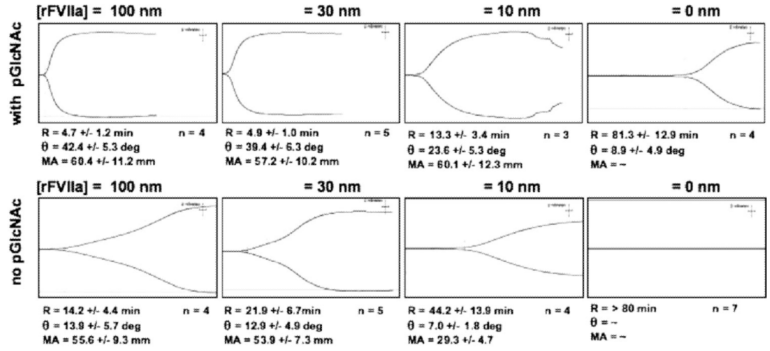

Figure 1.

pGlcNAc interaction with rFVIIa in FIX(−) canine blood affected clot formation. TEG analysis was performed in whole blood from hemophilia B dogs with the indicated concentrations of rFVIIa in the presence (*upper panels*) or absence (*lower panels*) of 1.0 mg/ml pGlcNAc. TEG parameters and average deviations are listed below each panel.

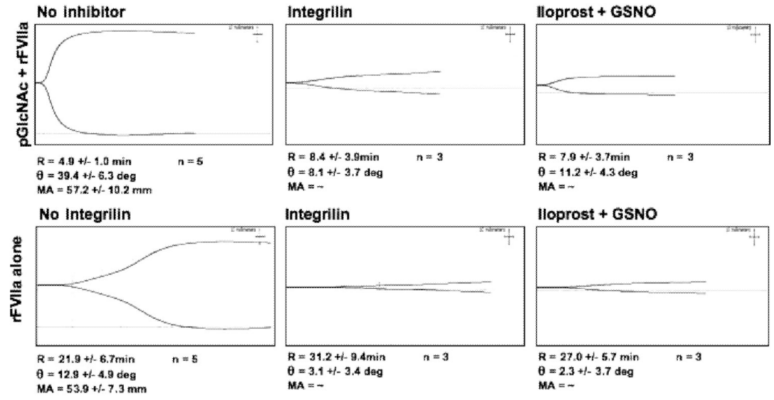

Figure 2.

rFVIIa and pGlcNAc interaction for clot formation in FIX(−) canine blood is affected by platelet inhibition. TEG analysis was performed in the presence (*upper panels*) or absence (*lower panels*) of 1.0 mg/ml pGlcNAc. Thirty nanometers of rFVIIa was included in all samples. Integrilin (50 μM) or a combination of Iloprost (10 μM) and GSNO (100 μM) was included during the TEG analysis. TEG parameters and average deviations are listed below each panel.

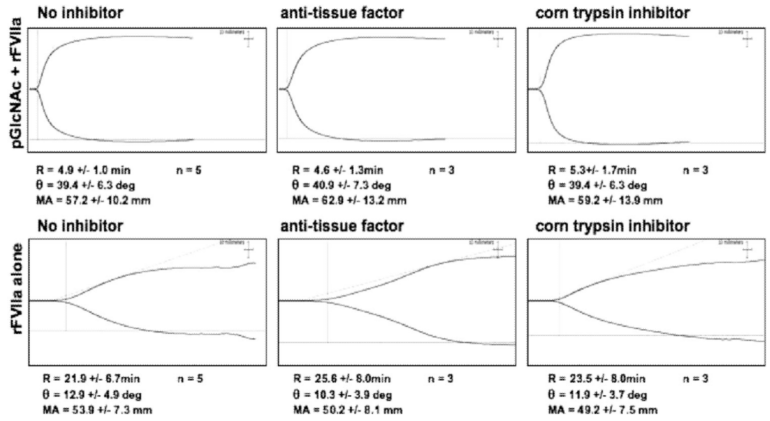

Figure 3.

Acceleration of clot formation by pGlcNAc and rFVIIa in FIX(−) canine blood is not affected by FVIIa or tissue factor. TEG analysis was performed in the presence (*upper panels*) or absence (*lower panels*) of 1.0 mg/ml pGlcNAc. Thirty nanometers of rFVIIa was included in all samples. Antitissue factor monoclonal antibody (50 nM) or corn trypsin inhibitor (50 units/ml) was included during the TEG analysis. TEG parameters and average deviations are listed below each panel.

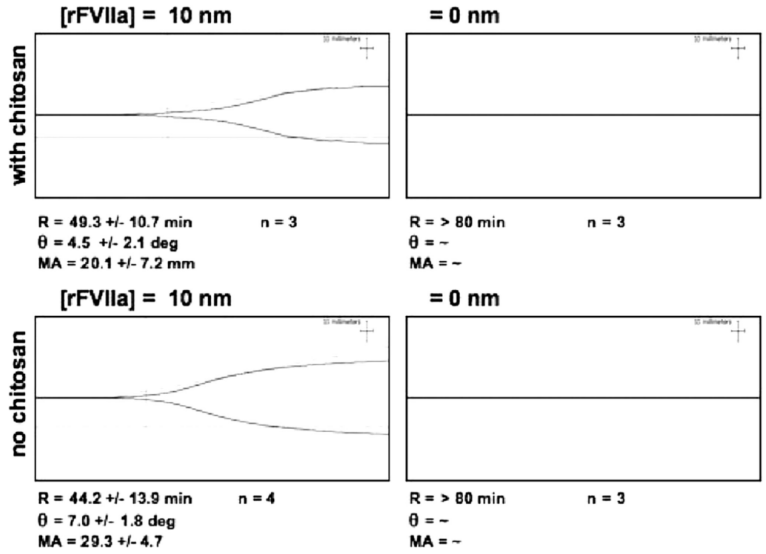

Figure 4.

Chitosan inhibits rFVIIa-mediated clot formation in FIX(−) canine blood. TEG analysis was performed with 10 nM rFVIIa in the presence (*upper panels*) or absence (*lower panels*) of 1.0 mg/ml chitosan in whole blood. TEG parameters and average deviations are listed below each panel.

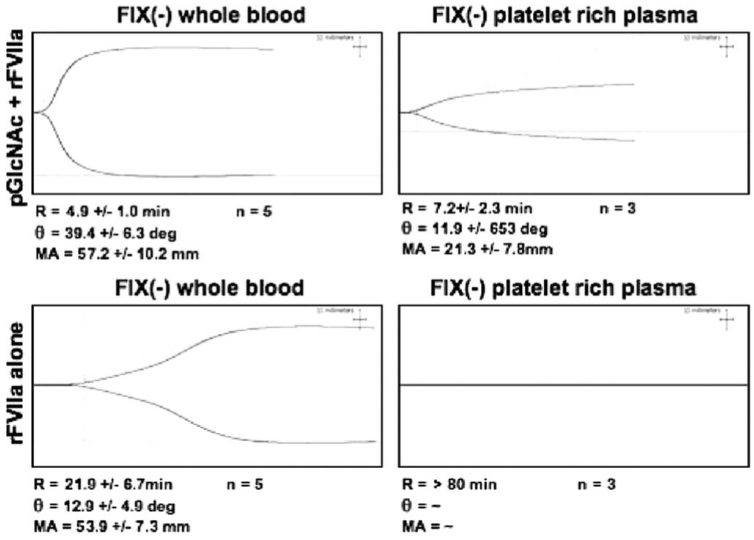

Figure 5.

pGlcNAc and FVIIa interaction to produce clot formation in the absence of FIX is more pronounced with RBCs. TEG analysis was performed with 30 nM rFVIIa in the presence (*upper panels*) or absence (*lower panels*) of 1 mg/ml pGlcNAc in whole canine blood or platelet-rich plasma. TEG parameters and average deviations are listed below each panel.