Recombinant Human Fibrinogen That Produces Thick Fibrin Fibers with Increased Wound Adhesion and Clot Density

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ABSTRACT: Human fibrinogen is a biomaterial used in surgical tissue sealants, scaffolding for tissue engineering, and wound healing. Here we report on the post-translational structure and functionality of recombinant human F1 (rF1) made at commodity levels in the milk of transgenic dairy cows. Relative to plasma-derived fibrinogen (pF1), rF1 predominately contained a simplified, neutral carbohydrate structure and >4-fold higher levels of the γ-chain transcriptional variant that has been reported to bind thrombin and Factor XIII. In spite of these differences, rF1 and pF1 were kinetically similar with respect to the thrombin-catalyzed formation of protofibrils and Factor XIIIa-mediated formation of cross-linked fibrin polymer. However, electron microscopy showed rF1 produced fibrin with much thicker fibers with less branching than pF1. In vivo studies in a swine liver transection model showed that, relative to pF1, rF1 made a denser, more strongly wound-adherent fibrin clot that more rapidly established hemostasis.

1. INTRODUCTION

Known as coagulation Factor I (F1), fibrinogen is a complex protein which polymerizes to form a wound adherent fibrin barrier that stops bleeding and acts as a scaffold for healing.²–⁴ During the healing process, the fibrin clot is enzymatically digested and reabsorbed.²–⁴ These characteristics make F1 naturally useful as a biomaterial in surgical tissue sealants⁵–⁷ and as an important tool in tissue engineering.⁸–¹¹ For decades, F1 has been used in the treatment of hemorrhage incurred on battlefields, in civilian trauma,¹² and in surgical procedures.¹³ The therapeutic transplantation of tissue made from autologous cells frequently uses a provisional matrix made of cross-linked fibrin to help culture and then deliver layers of cells into debrided wound sites.¹⁰ Because of its role in making fibrin polymer, F1 is a protein present at a high concentration of 2–4 g/L in human plasma,¹⁴ and its clinical applications typically use large 0.1–2 g doses.⁵ Unfortunately, a safe and abundant supply of plasma-derived F1 (pF1) is limited worldwide by the availability of pathogen-screened plasma.¹⁵,¹⁶ Surgical applications for tissue sealants¹³ in the United States indicate that F1 would need to be manufactured at amounts greater than several metric tons per annum (Supporting Information, Table S1).

F1 is a transcriptionally and post-translationally complex molecule. For example, after transcription of three separate genes, the human liver translates and assembles two Aα, two Bβ, and two γ-polypeptides into hexameric F1 having a molecular weight of 340 kDa.¹⁷,¹⁸ Assembly arises from the restrictive pooling of free chains within the endoplasmic reticulum prior to secretion as a holoprotein. In addition, there are two variations of the γ-chain. About 11% of pF1 contain a subpopulation of the γ-chain (γ′), which is a result of an alternative mRNA splicing event that replaces the four amino acid residues on the carboxy-terminal of the γ-chain with a 20 amino acid fragment.¹⁹,²⁰ The γ′ and γ subpopulations are both physiologically important.²¹–²⁴ The activation of F1, assembly into protofibrils and fibrin cross-linking are also affected by post-translational sulfation²⁵, phosphorylation,²⁶ and glycosylation.²⁷,²⁸ Importantly, the γ′-chain content and the post-translational modifications of F1 have been associated with opposing changes in fibrin fiber diameter, porosity, and degree of branching.²⁷,²⁹–³¹ Past reports show that the impact of increased γ′ content on fibrin formation is slowed fibrin polymerization, decreased fiber diameter with increased branching that produces smaller pores.²⁹–³¹ In contrast, in vitro deglycosylation of F1 produced fibrin structure with a larger fiber diameter, decreased branching, and larger pores.

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 Deglycosylation also resulted in increased polymerization rates but no change in the activation kinetics of FI.\textsuperscript{27} The physiologic significance of FI with an in vivo modified glycoform has not been reported. Indeed, there is currently no clear understanding between altering FI structure and the hemostatic barrier function at a wound site.

The molecular complexity of FI and commodity amounts needed for biotherapeutic applications are best addressed by the biosynthetic capabilities of mammalian cells. For example, classical large-scale suspension culture of animal cells can make hundreds of kilograms of therapeutic-grade, 2-chain assembled, humanized monoclonal recombinant antibody per year.\textsuperscript{32} However, an impediment to higher productivity in mammalian cell culture at the metric ton per year level is the low cell density of about 10^7 cells/mL of bioreactor media.\textsuperscript{53} As a result, the economics of scale-up necessitate large capital commitments of >$300 M in multiple bioreactors.\textsuperscript{2,34} Alternatively, the mammary gland of transgenic livestock can potentially make complex blood proteins in milk at rates of 2000 L/year per dairy animal.\textsuperscript{35} Compared to animal cell bioreactors, the cost of scale-up to add manufacturing the biosynthetic capabilities of mammalian cells. For example, the post-translational modifications made by the mammary epithelia relative to the human liver. The functional role of the rFI in vitro is examined by turbidimetric and viscoelastic properties as related to the formation of fibrin protofibrils and a cross-linked fibrin clot. We also study the physical structure of rFI fibrin by electron microscopy. The hemostatic function and wound adhesion of rFI is examined in an in vivo porcine hepatic injury model and by immunohistochemistry of the fibrin–wound interface.

2. EXPERIMENTAL SECTION

2.1. Materials. All reagents were obtained from Sigma unless otherwise specified. Plasma-derived human thrombin (pdHT), plasma-derived FI (pdFI) depleted of plasminogen, von Willebrand factor and fibronectin, and bovine fibrinogen (bFI) was bought from Enzyme Research Laboratories (South Bend, IN). Recombinant human thrombin (rHT) was purchased from ZymoGenetics (Seattle, WA). Anhydrous dimethyl sulfoxide (DMSO), sodium hydroxide, and methyl iodide were obtained from Sigma (St. Louis, MO). PNgase F was purchased from New England Biolabs (Beverly, MA).

2.2. Transgene Construction. The use of bovine αS1-casein promoter to direct mammary epithelial expression has been previously described.\textsuperscript{43} The complete 5.2 kbp human Act, 7.6 kbp bGp, and 8.5 kbp γ fibrinogen genes were cloned from a human genomic library contained within a P1-bacteriophage derived artificial chromosome (PAC) in Escherichia coli cells from Genome Systems, Inc. (St. Louis, MO). All three transgenes were constructed using the 6.2 kbp bovine αS1 casein S′-upstream promoter linked to the genomic fibrinogen coding sequences followed by each chain specific 3′ UTR. The following linearized transgene constructs resulted in a: 16.7 kbp S′-bovine αS1-casein, α fibrinogen gene (Supporting Information, Figure S1A); a 18.3 kbp S′-bovine αS1-casein, β fibrinogen gene (Supporting Information, Figure S1B); and 21.7 kbp S′-bovine αS1-casein, γ fibrinogen gene (Supporting Information, Figure S1C). These constructs were cotransfected into a female genital ridge cell line using calcium phosphate precipitation (CallPhos, Clontech, Mountain View, CA) to make founder animals by nuclear transfer.\textsuperscript{36,38}

2.3. Southern Blot Analysis. Genomic cow DNA was isolated from blood using Broodram procedure and subjected to restriction enzyme digestion with either BsrGI, HindIII or BglII as per the manufacturer’s instructions (New England BioLabs, Ipswich, MA). Digests were loaded on a 0.8% agarose gel and subjected to electrophoresis for 4 h at 120 V. The gel was washed in 0.25 N HCl for 15 min and then 0.5 N NaOH for 30 min before transferring to a MagnaCharge membrane (GE Healthcare, Uppsala, Sweden) using the Turbo blot system from BioRad (Hercules, CA). The αS1 casein probe was generated by PCR using Kirkegaard and Perry Laboratory’s (KPL) Detector Biotinylation kit (Gaithersburg, MD). The 500 bp probe corresponding to a region from 200 to 700 bp upstream from transcriptional start in the casein gene (S′ UTR promoter region) was used to detect the endogenous αS1 gene and each transgene transgene. After cross-linking, the membrane was probed and detected using the KPL AP-Chemiluminescent Blotting kit per manufacturer's instructions. Hybridization was carried out at 50 °C and high stringency wash at 60 °C.

2.4. Purification. rFI was purified from 32 L of milk collected on eight lactation days from two transgenic cows (Foxy and Fantasy) of the BFI2n8c83-EGFIneo lineage. Processed in 4L batches, rFI was purified by a two-column purification procedure using cation exchange (CIEX) and hydrophobic interaction chromatography (HIC). Transgenic milk was clarified, and loaded on a Butyl Sepharose (GE Healthcare, Piscataway, NJ) equilibrated in 20 mM sodium phosphate, pH 7.0. After loading, the column was washed with five volumes of the same buffer and bound proteins were eluted with a linear salt gradient from 0 to 0.5 M NaCl in 10 column volumes at a linear flow rate of 60 cm/h. Elution fractions containing rFI were pooled, diluted (1:1) with 1 M ammonium sulfate, and loaded on a Butyl Sepharose (GE Healthcare, Uppsala, Sweden) column (10/15 cm) equilibrated in 50 mM sodium phosphate pH 7.0 + 0.5 M ammonium sulfate. After loading and washing, the column was eluted with a linear salt gradient from 0.5 to 0 M ammonium sulfate in 10 column volumes at a linear flow rate of 60 cm/h. Elution fractions containing rFI were pooled, concentrated and buffer exchanged to 20 mM sodium citrate pH 7.0 + 0.15 M NaCl. Purified rFI from the 4L processing batches were pooled. The purified protein was subsequently filtered over 0.22 μm and stored at −70 °C until use. The step yields of the column purifications were ~60%; the overall yield of rFI was ~40%. Purity was evaluated by size exclusion chromatography. After being passed through a 0.20 μm polyester filter (Millipore, Billerica, MA), 0.5 m of purified rFI was passed through a TSK-G3000SWxL (Tosoh Biosciences, South San Francisco, CA) column (14 mL, 30 cm length, 7.8 mm ID) attached to a Knauer HPLC System at 0.5 mL/min for 45 min and data were collected by a photodiode array (PDA) with a 1 mm flow cell and analyzed by EZChrom Elite software.

2.5. SDS-PAGE and Western Blot. Nonreduced and reduced FI samples of purified pdFI, rFI, and bovine fibrinogen (bFI) and transgenic and nontransgenic milks were evaluated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 4–12% NuPage Bis-Tris gels (Invitrogen, Carlsbad, CA). Gels were then stained with Coomassie Blue (Invitrogen, Carlsbad, CA) or electroblotted onto Immobilon-Blot polyvinylidene fluoride (PVDF) membranes (BioRad, Hercules, CA) for immunoblotting. Blots were...
probed with a polyclonal antibody for human FI (USBiological, F4200—07C, Swampscott, MA).

2.6. γ- and γ'-Chain Content. FI samples (200 μg) were deglycosylated with peptide N-glycosidase F (PNGase F, New England Biolabs, Ipswich, MA), adjusted to 3 M GuHCl, reduced for 1 h with tris-(2-carboxyethyl)phosphine (TCEP, 5 mM final concentration), Pierce, Rockford, IL), alkylated for 30 min with iodoacetamide (15 mM final concentration), and then adjusted to 0.2% (v/v) formic acid. Analysis was performed on an Agilent 6210 ESI-TOF MS with an Agilent 1200 capLC using liquid chromatography coupled to electrospray time-of-flight mass spectrometry (LC-ESI-TOF-MS). The column was an Agilent Poroshell 300SB-C8 with dimensions of 0.5 mm ID and 7.5 mm L (Agilent Technologies, Santa Clara, CA). The flow rate was 20 μL/min, and the gradient program consisted of injection in 5% acetonitrile (LC-ESI-grade) in 0.1% formic acid followed by washing and then a linear gradient of 1%/min to 55% acetonitrile in 0.1% formic acid. MS data were acquired in positive mode. The raw data were deconvoluted with Agilent’s Qualitative Analysis software (v B.0.1.03) to generate the zero-charge spectra. The corresponding peak abundances were used to estimate the amounts of each species.

2.7. Fibrinopeptide Phosphorylation. Fibrinopeptides A (FpA) and B (FpB) were obtained by incubating rFI and pdFI (10 mg/mL) with rFla (236 U/mL) at 37 °C for 60 min. Phosphorylation was identified by a LC-MS/MS system which included a Dionex U3000 nanoflow HPLC system with a UV detector and an Applied Biosystems 4000 Q-TRAP triple quadrupole/ion trap mass spectrometer. The samples were injected onto a Dionex Acclaim Pepmap C18 trap column (Thermo Scientific, Rockford, IL) and the peptides were eluted by a linear gradient of 15—40% acetonitrile in 0.1% formic acid. Data were analyzed manually to confirm phosphorylation sites using theoretical m/z values calculated from the UCFS Protein Prospector MS-Product Web site and Analyst 1.4.2. The fibrinopeptide release chromatograms showed that the Fpa from pdFI consisted of two peaks: a fronting shoulder that accounted for ~20—30% of the peak area and the main peak. The fronting shoulder is the phosphorylated at Ser3. Comparison of the FI FpA elution time indicated that the majority of rFI was phosphorylated at Ser3. This was confirmed by LC-MS/MS analysis as described in Supporting Information.

2.8. Glycosylation Analysis. Sialylation profiling of rFI N-glycans was performed by normal phase high performance liquid chromatography (NP-HPLC) using the method of Anumula and Dhume.45 For ESI-MS/MS analysis of the glycans, rFI was incubated with PNGase F. Released N-glycans were separated from proteins using a C18 Extract Clean column (100 mg, 1.5 mL, Alltech, Deerfield, IL), eluted with 2 × 0.5 mL of solvent 0.1% (w/v) TFA in 50% acetonitrile/50% water, and dried by speed-vac (Labconco CentriVap, Kansas City, MO). The dried glycan solution was dissolved with a DMSO/NaOH suspension (100 μL) in 1.5 mL centrifuge tube and allowed to sit at room temperature for 30 min with occasional vortexing. Methyl iodide (50 μL) was added and the mixture was vortexed for 60 min. After the reaction, 100 μL of DMSO/NaOH suspension and 50 μL of methyl iodide were added again and vortexed for 60 min. A 500 μL aliquot of chloroform was added and washed repeated with ice-chilled water until the aqueous phase became neutral. The organic phase was dried under speed-vac for MS analysis. MS analysis was performed on a 4000 Q-Trap hybrid triple quadrupole/ion trap system (Applied Biosystems, Foster City, CA) with a Microlon Spray II ion source. Premethylated N-glycan was prepared in 70% acetonitrile/30% water. The sample solution was directly infused using a syringe pump at 0.3 μL/min. Possible structures were proposed by analyzing MS spectra and searching theoretical precursor ion mass on web-based Glycomod. The structures were determined by analyzing the corresponding MS/MS spectra and matching with calculated fragment ion mass consistent with the precursor ion definition using Glycoworkbench.46

2.9. Thrombin-Catalyzed Activation. The thrombin-catalyzed release of fibrinopeptides A (FpA) and B (FpB) from rFI and pdFI (Enzyme Research Laboratories, South Bend, IN) was determined based on the method from Gorkun et al.47 Two levels were used: (1) pdFI or rFI (0.1 mg/mL) incubated with rFla (0.01 U/mL) as described previously,47 and (2) pdFI or rFI (0.5 mg/mL) incubated with rFla (0.05 U/mL). Reversed-phase high-performance liquid chromatography (HPLC) was used to monitor fibrinopeptides on a Waters 2695 Alliance with a Waters 2996 PDA detector using a Jupiter C18 column (300 Å, 2 × 150 mm, 5 μm particles; Phenomenex, Torrance, CA). Waters Empower software was used to generate calibration curves ($R^2 > 0.996$ for Fpa and Fpb) using Agilent standards (Sigma, St. Louis, MO) and integrate peak areas for quantification. Data were plotted as percent release with respect to time assuming 100% release at 180 min. N = 6 for the low level condition (0.1 mg/mL FI and 0.01 U/mL thrombin) and N = 2 for the high level condition (0.5 mg/mL FI and 0.05 U/mL thrombin).

2.10. Thrombin-Catalyzed Proteofibril Formation. Polymerization of pdFI and rFI after treatment with thrombin was measured by changes in turbidity over time at 350 nm with a Beckman Coulter General Purpose spectrophotometer (Brea, CA), as previously described.47 pdFI or rFI (0.5 mg/mL) was loaded into a 10 mm optical path microwellcuvette. Thrombin (0.1 U/mL) was added. Samples were run in triplicate (N = 3). The change in turbidity was monitored at 350 nm for 30 min at 25 °C.

2.11. Factor XIIIa Catalyzed Molecular Cross-Linking. Activated recombinant factor XIIIa subunit (rFxiIIa) was produced in Pichia pastoris as previously described.46 The specific activity of the rFxiIIa was measured to be 7000 U/mg using Pefakit (Pentapharm, Norwalk, CT). pdFI (Enzyme Research, South Bend, IN) was depleted of constitutive FXIIIa by immunofinity purification using an antibody against FXIIIa monoclonal antibody (Green Mountain Antibodies, Burlington, VT). Cross-linking of FI by rFxiIIa was analyzed as previously described.47 rFI or pdFI (0.38 mg/mL) was incubated with rFxiIIa (1 U/mL) and four levels of rFxiIIa (1, 5, 25, and 100 U/mL) for 0, 2.5, 5, and 15 min at 24 °C. FI was also incubated with thrombin without rFxiIIa for 15 min, respectively. Cross-linking was studied by reducing SDS-PAGE (4—12% Bis-Tris NuPAGE) stained with Colloidal Blue (Invitrogen, Carlsbad, CA). The pdFI and rFI samples treated with rFxiIIa and 25 U/mL rFXIIIa for 15 min were electrophoresed onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) by applying 30 V for 1 h and stained with Colloidal Blue. The bands at approximately 130 and 150 kDa were excised and the first 10 amino acids in the N-terminal of each were sequenced by Edman degradation with an Applied Biosystems 494 Procise automated sequencer. N-terminal sequencing was performed by the University of Nebraska Medical Center’s Protein Structure Core Facility. The disappearance of the α-chain and γ-chain were analyzed by densitometry using Adobe Photoshop Elements 6.0 software.

2.12. Viscoelastic Characterization. Clot kinetics and strength was evaluated by thromboelastography (TEG) which was performed on solutions containing purified rFI or pdFI, rFxiIIa and rFxiIIa with a Thromboelastograph (TEG) Hemostasis System 5000 series (Hemoscope Corp., Niles, IL). rFI or pdFI (9 mg/mL) was incubated at 37 °C with rFxiIIa (53 U/mL), rFXIIIa (2429 U/mL), and CaCl₂ (12 mM). As a reference, normal citrated human blood (340 μL, N = 20 donors) was combined with 200 mM CaCl₂ (20 μL), as instructed by the TEG manufacturer. The TEG measured the strength of the clot as it formed. From those measurements, the TEG Analytical Software (version 4.2.2, Hemoscope, Niles, IL) calculated time to clot initiation (R), time to achieve a clot firmness of 20 mm (K), and maximal clot strength (MA), which is directly related to the shear elastic modulus strength (G).49,50 Each sample was run in triplicate (N = 3) so mean and standard deviation could be calculated. The data were exported and analyzed in Microsoft Excel.

2.13. Scanning Electron Microscopy. Scanning electron microscopy was utilized to examine the structure of fibrin clots formed by rFI and pdFI. FI (0.5 mg/mL) was incubated with rFxa (0.5 U/mL) for 1 h at room temperature. Samples underwent 2.5% glutaraldehyde fixation, stepwise ethanol and hexamethyldisilazane dehydration, and sputter coated with gold—palladium. Clots were imaged by a scanning electron microscope (S4700 Field-Emission SEM, Hitachi, Tokyo, Japan) at 15 kV and a magnification of 10000×. Average fiber diameters were measured from 20 fibers. The number of
branch points was determined in 10.4 μm² areas. Fiber diameters and branch points were statistically compared by t test with an α of 0.05.

2.14. Tissue Sealant Function. Crossbred (domestic) swine obtained from UNL Agricultural Research and Development Center (Mead, NE) were anesthetized with isoflurane (1–2%) supplemented with oxygen (1–2 L/min) throughout the procedure. A carotid arterial catheter for pressure monitoring and blood sampling and a jugular venous catheter for fluid and medication administration were placed via surgical cutdown in the left neck. Blood samples from each pig were drawn before, during, and after surgery and tested by thromboelastography (TEG) to identify and select pigs with normal clotting parameters and to monitor changes in endogenous coagulation parameters throughout surgery due to blood loss or hemodilution by Lactated Ringers solution. The liver was exposed through a midline incision. A scissors-shear injury clamp⁵¹–⁵³ with X-shaped blades (5 cm width and breadth) was applied through the central portion of the liver, adjacent to the vena cava, as described previously.¹² The resultant injury was a jagged, stellate laceration 8–10 cm in diameter, completely through the organ. Such injuries were treated in 10 pigs with LFS (7 mg/mL pdFI or rFI (N = 1 and 9, respectively), 1740 U/mL rFXIII, 85 U/mL rFIIα, 12 mM CaCl₂; total of 6 mL) applied with a Tissel Duploject dual-syringe system (Baxter Healthcare Corporation) over 3 s to several minutes followed by 3–5 min of manual compression. Euthanasia was performed while the animal was under deep isoflurane anesthesia. Sodium pentobarbital (380 mg/mL, 10 mL IV) was administered, and 1 min later the animal underwent bilateral diaphragm incisions with transection of the supradiaphragmatic vena cava and aorta. All procedures performed during this research were approved by the Institutional Animal Care and Use Committee of the Omaha VA Medical Center.

2.15. Immunohistochemistry. Adherence of the human fibrin made from rFI and pdFI to wounded tissue was examined by immunohistochemistry. Wedge-shaped hepatic excisions (base of 1 cm and height of 1 cm) were made along a lobar edge. Fibrin sealant (FS), consisting of 9 mg/mL FI (pdFI or rFI), 2460 U/mL rFXIII, 106 U/mL rFIIα, and 12 mM CaCl₂, was applied by spray device to the wound for about 35 s. Approximately 18 mg FI, 5000 U rFXIIIa, and 210 U rFIIα was applied to each wedge. Liver sections from untreated and rFI and pdFI sealant-treated wedge excisions were fixed in 10% neutral-buffered formalin, dehydrated, and embedded in paraffin. Specimens were sliced (5 μm), mounted on slides, dewaxed, and processed with Dako TRS antigen retrieval solution (Dako, Carpinteria, CA). After blocking endogenous alkaline phosphatase and peroxidase activity, the specimens were incubated with an antiporcine fibrinogen antibody (Kamiya Biomedical Company, Seattle, WA) and exposed with HRP/DAB⁺. After blocking remaining peroxidase activity associated with the antiporcine fibrinogen antibody, specimens were incubated with an antihuman fibrinogen antibody (Abcam, Inc., Cambridge, MA) and exposed by alkaline phosphatase-based Permanent Red (Dako, Carpinteria, CA). The specimens were subsequently counterstained with Mayer’s hematoxylin. DAKO EnVision G2 Doublestain System (Dako, Carpinteria, CA) was used for signal detection.

3. RESULTS

3.1. Somatic Cell Transfection and Nuclear Transfer. The characterization of the transgenic cow clonal lineage BFI2n8c83-EGFIneo (termed “Fancy”) is presented here as an example of a stable, cloned α-S1 casein-FI genotype. Using Southern analysis, we characterized the presence of each of the three α-S1 casein-FI transgenes (Supporting Information, Figure S1D) in Fancy’s DNA isolated from leukocytes harvested from whole blood. Three restriction endonuclease digestions were chosen to yield fragments containing a common element of the α-S1 casein promoter while having differently sized sequences unique to each fibrinogen gene. The specificity of the probes for each of the BsrGI, HindIII, and BfII digests of Fancy’s and control bovine DNA was demonstrated by the presence of endogenous casein signals in both. The control DNA lacked signals associated with the human FI sequences of the reference transgene mixture. In contrast, Fancy’s DNA contained hybridization signals specific to the digests of the reference α-S1 casein rFI transgene mixture and eight or more copies of each Act-β, Bβ, and γ-transgene. Two other cows of the Fancy clonal lineage (Foxy and Fantasy) showed similar copy numbers and Southern analysis results.

3.2. rFI Assembly, Concentration, and Purification. Fancy’s milk was collected and skimmed, and the resulting skimmed milk was examined using Colloidal blue stained SDS-PAGE and Western analysis under nonreducing (Figure 1A,B, lane 8) and reducing (Figure 1C,D, lane 8) conditions. We estimated the concentration of assembled rFI in the milk to be 2–4 g/L in three cows of the Fancy clonal lineage using densitometric analysis of Western blots with human pdFI as a reference. This level of production was stable for a lactation study conducted over a period of 200 days. Western analysis using a polyclonal antibody confirmed the identity of the main 340 kDa species as assembled human FI. The Mₙ of the assembled fibrinogen (340 kDa) is indicated by closed arrows in the upper panels and each individual Act-β, Bβ, and γ-chains of FI are indicated by α, β, γ, and γ’, in the lower panels. The open arrow indicates free Act-β.

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The Act-chain of pdFI and rFI appeared as doublet bands, which has been reported previously for pdFI. SDS-PAGE partially resolved γ- and γ′-chains (Figure 1C, lane 4). The hexameric nature of pdFI and rFI was corroborated by the 340 kDa M, under nonreducing conditions. Some smaller amounts of FI reactive species with higher and lower M, than the 340 kDa band were present in the skimmed milk (Figure 1B, lane 8). Some of these species were similar to those found in pdFI (Figure 1B, lane 2) and thus could have been unassembled Act-chains.

For the analysis presented here, approximately 38 g of rFI was purified from 32 L of milk from eight lactation days of two transgenic cows (Foxy and Fantasy). As detected by electrophoresis, rFI was purified to a single species using a two-step sequence of cation exchange (CIEX) and hydrophobic interaction chromatography (HIC). The purity was estimated to be >98% (Figure 1A,C, lane 4 and Figure S2, Supporting Information) with a yield of >40%. Purified rFI was examined for the presence of bovine fibrinogen (bFI). Differences in the molecular size of assembled pdFI, bFI and rFI samples were readily discriminated from each other using SDS-PAGE under reducing conditions (Figure 1C). The side-by-side comparison of the individual FI chains using electrophoresis and LC-ESI-TOF-MS showed no discernible contamination of bFI in the rFI sample. The bFI content in this sample is likely to be <2%. Taken together, rFI was sufficiently isolated from other milk proteins enabling the characterization of its structure and related biological activity in vitro and in vivo.

3.3. γ- and γ’-Chain Content. The γ’ content of rFI was investigated using mass spectrometry on deglycosylated, reduced, and alkylated samples. The theoretical molecular weight (MWavg) for the γ and γ′ chains after this sample treatment is 47040 and 49054 Da, respectively, with each sulfation adding 80 Da to the molecular weight (MWavg) for the γ and γ′ chains. In both pdFI (Figure 2A) and rFI (Figure 2B), the γ-chain was the major translated species (extra carbamidomethylation of the γ-chain, an artifact of the sample processing, is seen in both pdFI and rFI). The γ′-chain of pdFI is sulfated at Tyr418 and/or Tyr422. The C-terminal γ′ peptide has two potential sites of Tyr-sulfation. pdFI essentially had all doubly sulfated γ′ (theoretical molecular weight 49214 Da). We observed evidence of partial sulfation within the γ′-chain of rFI (theoretical molecular weight 49134 Da). Accounting for all observed forms of the γ- and γ′-chains in the deconvoluted spectra, there was >4-fold higher γ′ in rFI than the pdFI reference.

3.4. rFI Biochemical Features. Phosphorylation, sulfation, and glycosylation of rFI were analyzed by HPLC and LC-MS. The degree of phosphorylation of fibrinopeptide A (FpA) of rFI and reference pdFI after treatment with thrombin was evaluated by reverse phase C-18 HPLC (Figure S3A) and confirmed by LC-MS/MS (Figure 3B). Greater than 90% of the transgenic FpA was phosphorylated in contrast to 20–30% phosphorylation of FpA from the pdFI reference.

Figure 3. Fibrinopeptide phosphorylation of rFI. (A) Phosphorylation of fibrinopeptides. FpA and FpB released into the supernatant from fibrin clots formed by the treatment of pdFI and rFI (10 mg/mL) with thrombin (236 U/mL) were analyzed by C-18 HPLC and LC-MS as described in the experimental protocol. Synthetic FpA and FpB peptides were used as nonphosphorylated reference standards. Peak assignment is indicated above the corresponding peaks. (B) MS/MS spectrum of FpA released from thrombin treatment of rFI, confirming phosphorylation at Ser3. In CID, phosphorylated serine residues undergo a characteristic neutral loss of H3PO4 (98 amu). This characteristic loss is evident for the precursor ion ([M + 2H − H3PO4]2+ = 759 m/z), the b fragment ions starting with b1 (b1, H2PO4 = 256 m/z), and the y1 (H3PO4 = 1332 m/z). These results lead to the conclusion that Ser3 is phosphorylated.

Glycosylation is a primary determinant of the clearance of glycoproteins in circulation by the liver. Potential sites of N-glycosylation in pdFI and rFI are at Asn364 of the B′-chain and Asn42 of the γ-chain where the glycans in pdFI are biantennary complex glycans with one or two sialic acids. Glycosylation of the purified rFI samples was investigated by normal phase HPLC profiling and mass spectrometry. The results of the HPLC profiling for pdFI were consistent with biantennary glycans.
complex glycans with one or two sialic acids (Figure 4A); however, the majority of the N-linked glycans in rFI were neutral structures (Figure 4B). N-glycans were enzymatically released from rFI and permethylated for MS and MS/MS analysis. The N-glycans of rFI were a mixture of high mannose, neutral complex, and hybrid glycans (Figure 4C). These structures displayed a whey protein glycan signature similar to those observed with bovine lactoferrin. A small amount of rFI N-glycans containing one sialic acid likely were degraded during permethylation. In summary, the post-translational modifications of rFI displayed a milk protein signature which was different than that of pdFI.

3.5. Activation Kinetics. The thrombin-catalyzed release of fibrinopeptides A (FpA) and B (FpB) was monitored by HPLC. We studied FpA and FpB release using previously reported FI and thrombin concentrations and at 5-fold higher FI and thrombin concentrations to compare pdFI and rFI activation kinetics. At both levels, FpA and FpB release kinetics were similar for pdFI and rFI in that both showed the slower release of FpB relative to FpA. Hence, the activation of rFI and pdFI were similar.

3.6. Fibrin Protofibril Formation. The rates of protofibril formation of pdFI and rFI activated by thrombin in the absence of added FXIIIa were measured by the change in turbidity (Figure 6). At 0.2 mg/mL FI and 0.1 U/mL recombinant thrombin (rFIIa), the initiation of protofibril formation as measured by the time to initial onset of turbidity was slightly faster for pdFI than rFI. The rate of increase in turbidity, which is indicative of protofibril assembly and final absorbance, which is indicative of fiber diameter, were similar for pdFI and rFI.

3.7. Molecular Cross-Linking. We investigated the time course of polymerization of rFI and pdFI treated with rFIIa (1 U/mL) and rFXIIIa (25 U/mL) by SDS-PAGE analysis under reducing conditions. An insoluble fibrin clot, which dissolved after treatment with reducing agent and SDS at 74 °C, was observed in all samples treated with rFXIIIa. The normal constitutive level of FXIII contamination present in pdFI preparations was observable by the appearance of γγ dimers after treatment by rFIIa alone (Figure 7A, lane 8). In contrast, the absence of FXIIIa activity in the rFI was evident as no γγ dimers were formed after the addition of rFIIa alone (Figure 7B, lane 8). A total of 2.5 min after treatment of pdFI or rFI with rFIIa, we observed a 1.5 kDa shift to lower M of the Aα- and Bβ-chains, indicating a nearly complete conversion to their activated counterparts resulting from the release of FpA and FpB (pdFI, Figure 7A; rFI, Figure 7B). Densitometric

Figure 4. Biochemical features of rFI. N-glycans released by PNGase F from purified samples of pdFI and rFI were analyzed by HPLC (A, B) and mass spectrometry (C). (B, C) Elution times of neutral, monosialylated, and disialylated species are indicated.

Figure 5. Activation kinetics of pdFI and rFI. The time course of the release kinetics of FpA and FpB was analyzed by HPLC at two levels: (A) rFI and pdFI (0.1 mg/mL) incubated with thrombin (0.01 U/mL), and (B) rFI and pdFI (0.5 mg/mL) incubated with thrombin (0.05 U/mL); N = 6 (A) and N = 2 (B).

Figure 6. Fibrin protofibril formation of pdFI and rFI. Polymerization of pdFI and rFI at 0.2 mg/mL, initiated at time 0 with thrombin 0.1 U/mL, was monitored as the change in turbidity at 350 nm for 30 min (N = 3).
analysis indicated that the rates of disappearance of the β-chain were similar for pdFI and rFI (−0.16/min and −0.15/min, respectively; Supporting Information, Figure S3A) when rFXIIIa was added exogenously. Over the initial 5 min incubation period, the rate of α-chain polymerization was slightly faster for rFI than pdFI as shown by densitometry (−0.03/min and −0.06/min, respectively; Supporting Information, Figure S3B). Similar polymerization patterns were observed for pdFI and rFI. N-terminal sequencing indicated that the band at approximately 130 kDa was an α-chain multimer (Figure 7A,B, open arrow) and the band at approximately 150 kDa was a multimer (Figure 7A,B, open arrow) and the band at approximately 130 kDa was an αβγ multimer. Thromboelastographic comparison of rFI- and pdFI-based tissue sealants (C). TEG analysis of the kinetics of clot initiation and clot strength over time for pdFI and rFI (9 mg/mL) after treatment with rFIIa (53 U/mL) and rFXIIIa (2429 U/mL). N = 20 for normal human blood and N = 3 for the pdFI and rFI treatment groups.

3.8. Viscoelastic Properties. The function of fibrin as a barrier to bleeding can be related to its viscoelastic strength. The evolution of viscoelasticity during the formation of a cross-linked fibrin clot from rFI and pdFI were compared by TEG (Figure 7C). We used an optimized tissue sealant formulation of rFI, rFXIIIa, and rFI or pdFI to study the differences in clot strength and viscoelastic kinetics. The average viscoelastic behavior of 20 individual human blood samples strongly contrasts the rapid clotting behavior and high strength of both rFI and pdFI. The time to clot initiation was similar for rFI (18 ± 6 s) and pdFI (20 ± 5 s) (p = 0.64, α = 0.05). The maximum viscoelastic strength of the developing clot was about 65 mm (9573 ± 1115 dyn/cm²) for pdFI and about 63 mm (8637 ± 867 dyn/cm²) for rFI (p = 0.32, α = 0.05). In summary, both the kinetics of cross-linked fibrin clot formation and the clot strengths were equivalent for rFI and pdFI.

3.9. Scanning Electron Microscopy. The structure of clots formed by pdFI and rFI were compared by scanning electron microscopy (Figure 8). The fibrin from rFI had thicker fibers with larger pores and less branching than the fibrin from pdFI (Table 1). The average fiber diameter of the pdFI clot (0.10 ± 0.02 μm) was similar to diameters measured previously.30,31 The average fiber diameter of the rFI clot (0.19 ± 0.04 μm) was significantly greater than that of pdFI (p < 0.001, α = 0.05). In contrast, the rFI fibrin clot had a significantly lower proportion of branch points per area than the pdFI fibrin clot (3.0 ± 1.4 vs 6.0 ± 1.9; p < 0.001, α = 0.05).

Table 1. Analysis of Fiber Diameter and Number of Branch Points from SEM

<table>
<thead>
<tr>
<th>Fiber Diameter (μm) n = 20</th>
<th>pdFI Mean (SD)</th>
<th>rFI Mean (SD)</th>
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<tr>
<td>0.10 ± 0.02</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>0.19 ± 0.04n</td>
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n = 20 for normal human blood and n = 3 for the pdFI and rFI treatment groups.

3.10. Tissue Sealant Function. Because rFI had molecular and coagulation properties in vitro, similar to those of pdFI, we evaluated the wound adherence properties of fibrin made by rFI applied to a wound in vivo. All of the pigs maintained similar thromboelastographic clotting parameters before, during, and after surgery. rFI or pdFI was applied as a tissue sealant to a severe hemorrhage model generated from a grade V liver laceration involving the central hepatic veins.12 The grade V liver laceration generated a jagged wound topography with severely irregular edges up to 8 mm in diameter (Figure 9A). Untreated, this wound is a lethal, exsanguinating injury.12 The pdFI and rFI tissue sealant was applied subsurface into the pool of blood which obscured the wound. Even in the presence of diluting amounts of blood, the tissue sealant rapidly formed an adherent clot after five minutes of manual compression. While both the pdFI-based (Figure 9B) and rFI-based (Figure 9C) tissue sealant had thicken...
sealant rapidly formed an adherent barrier that stopped bleeding, qualitatively we observed that treatments with pdFI sealant became permeated with porcine fibrin and formed translucent clots, whereas the rFI treatments were less permeable and formed more opaque clots.

**3.11. Immunohistochemistry.** We examined the clotted wound surfaces by histological cross-sectioning and immunostaining for human and porcine FI (Figure 10A–C). While the human fibrin from pdFI was well intermixed with porcine fibrin (Figure 10B), the fibrin from rFI created well delineated layers alternated with porcine fibrin (Figure 10C). We observed a relatively uniform, 100 μm thick human fibrin layer on the outer surface of the rFI sealant treated tissue. Thus, both gross visual and histological observation of the wound treated with rFI-based sealant confirmed that the resulting fibrin clot was wound adherent and intercalated with blood-borne fibrin to produce a sufficiently strong barrier to bleeding.

**4. DISCUSSION**

Our studies provide the first illumination of the in vivo adhesive structure and hemostatic behavior of recombinant FI. Importantly, the molecular complexity of FI dictates that any rFI will be a facsimile and not an exact copy of the structure and function of pdFI. For example, our rFI is perturbed by increased γ′-chain content as well as differences in the carbohydrate structure at native sites within its α-chain. Importantly, these two particular structural determinants of FI have been associated with opposing changes in fibrin fiber diameter, porosity, and degree of branching.27,29–31 Past reports showed that increased γ/γ′ heterodimer content slowed fibrin polymerization, decreased fibrin diameter, and increased branching resulting in smaller pores.29–31 In contrast, deglycosylation of pdFI produced fibrin structures with larger fiber diameter, decreased branching, and larger pores. Deglycosylation also resulted in increased polymerization rates but no change in the activation kinetics of pdFI.27 Prior to this work, the physiologic significance of a post-translationally modified glycoform structure has not been reported. In addition, past work has provided no clear understanding between altering FI structure and the hemostatic barrier function that occurs at a wound site. Our work shows that changes in rFI structure might result in fibrin with improved hemostatic properties achieved without adverse effects on the speed of fibrin formation.

Our studies of rFI made in the milk of cows perturbs opposing determinants of fibrin hemostatic structure and function while making sufficient amounts for preclinical studies. We observed that the bovine mammary gland performed the same alternative mRNA splicing of the FI γ gene as the human liver resulting in the expression of the γ′ variant.19 However, the level of the γ′-chain population in rFI was strikingly higher than in pdFI. In addition, the mammary tissue also glycosylated the rFI with a neutral carbohydrate that resembled those of whey and casein milk proteins but not the ionically more bulky moiety displayed by FI made in the human liver. Surprisingly, in spite of its >4-fold higher γ′ content, the resulting rFI produced fibrin with thicker fibers, less branching, and larger pores compared to pdFI. Importantly, this was accompanied by no observable slowing in the molecular kinetics of fibrin assembly. This is a similar result for fibrin made from deglycosylated pdFI having much lower levels of γ/γ′. This prior work showed that removing the charged and branched carbohydrate structure sterically affected individual profibril alignment during the assembly into fibers.27,28 Thus, we conclude that the perturbation to a less bulky glycoform made by the mammary gland biochemistry more strongly
influenced fibrin structure over that of the $\gamma/\gamma'$ heterodimer content.

Previous studies have given conflicting data on the effect of changes in the overall phosphorylation of pdFI where both increased and decreased phosphorylation of pdFI were correlated with increased fiber thickness. However, these early studies did not elucidate the location of the phosphorylation within the pdFI and whether it occurred on the FpA activation peptide and at other alternate phosphorylation sites that may affect fibrin structure. Here, the phosphorylation of the rFI was 3-fold higher than pdFI and it occurred exclusively from increased phosphorylation of the FpA activation peptide, which is removed upon activation. The higher FpA phosphorylation in rFI likely reflects the very efficient phosphorylation of these same motifs in milk caseins. Perhaps more importantly, both FpA/FpB release kinetics and early phase turbidity kinetics were similar for pdFI and rFI. These kinetics are associated with the start of prototibril formation and are in agreement with past studies. Thus, we conclude that there was little observable impact by the higher phosphorylation that occurred in rFI.

Our porcine hepatic injury model consisted of astellate laceration imposed under normal coagulation potential. It produced severe solid organ hemorrhage primarily from venous blood flow and a rough wound topography of exposed collagen surfaces to examine fibrin adherence. While having equivalent thromboelastic kinetics and strength, as measured in vitro, the pdFI-based tissue sealant made a translucent and less hemostatic clot than the rFI when applied to a wound surface. In contrast, the fibrin generated from rFI made an opaque white and adherent clot that more rapidly resulted in hemostasis. Furthermore, the histology of the wounds treated by pdFI and rFI showed a strong contrast in clot structure leading to hemostasis: the fibrin from the rFI was adherent as a dense stratum less than 100 μm thick while the pdFI was well mixed into the wound and diluted with endogenous pig fibrin to a depth of about 300 μm or more. This is consistent with our macroscopic observation that the rFI clot was not permeated by red blood cells, while the pdFI clot was blood red in color. This reflects the different fiber structure seen in our SEM results and is likely caused by the presence of the stericly less bulky carbohydrate that is the signature glycosylation pattern of mammary tissue. Based on the wound adherence and hemostatic characteristics of rFI, we are currently conducting preclinical studies that will statistically compare the efficacy of a rFI-based tissue sealant to commercial-grade tissue sealants in a cold hemodilution, hypocoagulopathic grade V+ porcine liver laceration imposed under normal coagulation potential. It was fully assembled and produced at 2–4 g/L for a lactation study conducted over a period of 200 days. Despite differences in $\gamma'$ content, glycoform, and phosphorylation content of FpA, the kinetic and viscoelastic attributes of fibrin formation by rFI were similar to those by pdFI. The fibrin from rFI had significantly thicker fibers and a lower proportion of branching than clots from pdFI. We then used this material as a tissue sealant to study the function of fibrin made from rFI in a porcine hepatic injury model. In two different swine liver surgical trauma models, rFI formed a more opaque, histologically dense, wound-adherent fibrin clot that more rapidly stopped bleeding than pdFI.

5. CONCLUSION

This study describes the production, purification, and molecular characterization of large amounts of rFI produced in the milk of transgenic dairy cows. The rFI produced by transgenic cows was fully assembled and produced at 2–4 g/L for a lactation study conducted over a period of 200 days. Despite differences in $\gamma'$ content, glycoform, and phosphorylation content of FpA, the kinetic and viscoelastic attributes of fibrin formation by rFI were similar to those by pdFI. The fibrin from rFI had significantly thicker fibers and a lower proportion of branching than clots from pdFI. We then used this material as a tissue sealant to study the function of fibrin made from rFI in a porcine hepatic injury model. In two different swine liver surgical trauma models, rFI formed a more opaque, histologically dense, wound-adherent fibrin clot that more rapidly stopped bleeding than pdFI.

## Associated Content

### Supporting Information

1. Schematic illustrations of the three separate FI transgenes used in making the transgenic cows and a corresponding southern blot; (2) flow diagram of the purification process of rFI from milk and purity of rFI by size exclusion chromatography. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare the following competing financial interest(s): The academic institution of W.H.V. has licensed recombinant fibrinogen technology to Pharming Group NV. W.H.V. has no financial interest in Pharming Group NV. M.G., H.V.V. and K.N. are employees of Pharming Group NV.

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### References
