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ORIGINAL ARTICLE

Carbohydrate-binding activities of coagulation factors fibrinogen and fibrin

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Abstract The coagulation factors fibrinogen and fibrin play important roles in the final stage of the blood coagulation cascade. It has not been revealed whether fibrinogen has lectin activity or not. Here we demonstrate that fibrinogen and fibrin have carbohydrate-specific binding activities that inhibit fibrin clot formation. A solid-phase binding study using sugar-biotinyl polymer probes revealed that fibrinogen has the highest affinity to mannose (Man) in both the presence and absence of 5 mM Ca^{2+}. Fibrin, which is proteolytically produced from fibrinogen by thrombin, binds to the same sugar residues as fibrinogen in the presence of 5 mM Ca^{2+}, while it markedly binds to *N*-acetylneuraminic acid in the absence of Ca^{2+}. Thrombin-induced fibrin polymerization was monitored by turbidity at 350 nm. In the presence of Ca^{2+}, Man and sugars having *N*-acetyl groups were found to inhibit the increase in turbidity, but only Man inhibited it in the absence of Ca^{2+}. Scanning electron microscopy observation of fibrin clots formed in the presence of various sugars showed that fibrin fibers formed in the presence of Man and *N*-acetyl group sugars were thinner and more branched. In contrast, thrombin has neither carbohydrate-binding activity nor is affected by sugars. These results suggest that carbohydrates and glycoconjugates may regulate fibrin clot formation *in vivo*.

Keywords Carbohydrate-binding protein · Coagulation factor · Fibrin · Fibrinogen · Fibrin clot

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Abbreviations

TBS	20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl
TBS+Ca^{2+}	TBS containing 5 mM $CaCl_2$
TBS+EDTA	TBS containing 5 mM EDTA
TBS+EGTA	TBS containing 5 mM EGTA
PBS	10 mM phosphate buffered saline, pH 7.5
Sugar-BP	Sugar-biotinyl polymer
DL-BAPA	Benzoyl-DL-arginine *p*-nitroanilide hydrochloride
SEM	Scanning electron microscopy

Introduction

The blood coagulation cascade is regulated by a complex mechanism, but details of the activation mechanism and interactions among coagulation factors have not yet been completely revealed. Fibrinogen, a glycoprotein that is present at a concentration of 1.5 to 3.5 mg/mL in human plasma, circulates as the precursor of fibrin, a major structural component of the blood clot [1]. It has been reported that fibrinogen binds to glycosaminoglycans such as heparin [2] and hyaluronan [3]. However, neither fibrinogen nor fibrin is known to possess carbohydrate-binding activity toward neutral and acidic monosaccharides.

In this study, we discovered a novel carbohydrate-binding activity of fibrinogen and that several carbohydrates specificity changes with the conversion of fibrinogen to fibrin, which is a different mechanism from that of heparin and hyaluronan. Here we report the monosaccharide-binding activity and demonstrate the effect of carbohydrates on the polymerization of fibrin in relation to its biological function.

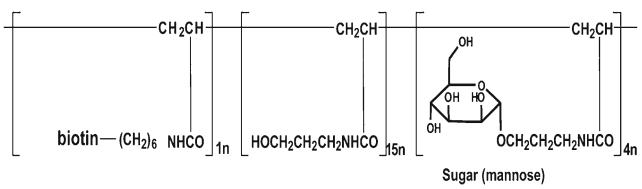
Materials and methods

Materials

Human fibrinogen was purchased from Enzyme Research Laboratories (South Bend, IN) and dialyzed with 20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl and 5 mM EDTA (TBS+EDTA) to remove Ca^{2+}, followed by 20 mM Tris buffer, pH 7.4, containing 0.15 M NaCl (TBS) at 4 °C before analysis. This fibrinogen preparation was contaminated by small amounts of factor XIII, as judged by the detection of bands of α-polymer and γ-γ cross-linked chains on SDS-PAGE under reducing conditions after the fibrinogen had been clotted with thrombin as described previously [4]. Lyophilized thrombin powder from bovine plasma was purchased from Sigma Chemical Co. (St. Louis, MO). Sugar-biotinyl polymer (Sugar-BP) probes were purchased from GlycoTech Co. (Gaithersburg, MD). Methyl α-D-mannopyranoside (Me α-Man), *N*-acetyl-D-galactosamine (D-GalNAc), *N*-acetyl-D-glucosamine (D-GlcNAc), *N*-acetylneuraminic acid (NeuAc), methyl β-D-glucopyranoside (Me β-Glc) and chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) or Wako Pure Chemicals Inc. (Osaka, Japan). Benzoyl-DL-arginine *p*-nitroanilide hydrochloride (DL-BAPA) was purchased from Peptide Institute, Inc. (Osaka, Japan).

Binding studies with sugar-BP probes

Binding to sugar-BP probes was measured by ELISA according to the procedure described previously [5]. The sugar-BP probe is a polyacrylamide polymer of approximately 30 kd containing 5%mol biotin and 20%mol carbohydrate. A schematic structure of the probe is shown in Scheme 1. Fibrinogen (0.625–10 μg/mL) and thrombin (1–15 μg/mL) were immobilized at 4 °C overnight in TBS (100 μL) on well of an Immulon 1B 96-well microtiter plate (Thermo Fisher Scientific Inc., Wayne, MI). All other procedures were performed using TBS or TBS containing 5 mM $CaCl_2$ (TBS+Ca^{2+}) as the dilution buffer. The wells were blocked with 3 % skim milk for 1 h at room temperature after immobilization. The incubation temperature with sugar-BP probes was 4 °C. To convert fibrinogen into fibrin, immobilized fibrinogen was treated with thrombin (1 U/mL) and aprotinin (400 U/mL) in 100 μL TBS or TBS+Ca^{2+} at 37 °C for 1 h after immobilization of fibrinogen and before blocking [6].



Scheme 1 Structure of sugar-BP probe used in this study

Fibrin polymerization and cross-linking

The polymerization profiles were monitored at A_{350} [7, 8]. Briefly, fibrinogen (5 mg/mL, 40 μL) in TBS or TBS+Ca^{2+} was preincubated with various sugars (0.2 M in the same buffer, respectively, 50 μL) at 37 °C for 5 min in a 96-well microtiter plate. Thrombin (0.5 U/mL, 10 μl in the same buffer) was mixed and monitored at A_{350} and 37 °C for 120 min with a microplate reader (Vient, DS Pharma Biomedical, Osaka, Japan). To examine the degradation of fibrinogen and cross-linking of fibrin, the reactions were started in plastic tubes and stopped at various times by adding the SDS sample buffer for gel electrophoresis (final 2 % SDS, 10 % glycerol, 0.0625 M Tris–HCl (pH 6.8), and 0.001 % bromophenol blue) [9] containing 1 % 2-mercaptoethanol and boiled at 98 °C for 10 min. The samples were subjected to SDS-PAGE on a 7 % polyacrylamide gel and stained with Coomassie brilliant blue R-250. The ratio of α-polymers per total band was calculated using ImageJ 1.47 (National Institutes of Health, Bethesda, MD).

Scanning electron microscopy (SEM) of fibrin clots

Fibrinogen (5 mg/mL, 80 μL) and various sugars (0.2 M, 100 μL) were mixed in TBS+Ca^{2+}, and thrombin (0.5 U/mL, 20 μL) was added. The mixture was quickly transferred to a glass vial (5×0.2 cm). Fibrin clots were formed by incubation at 37 °C for 90 min. The resulting fibrin clots were washed by 10 mM phosphate buffered saline, pH 7.5 (PBS) and fixed in 2.5 % glutaraldehyde overnight at 4 °C. The clots were rinsed in PBS and distilled water before being fixed at 4 °C for 1 h with 1 % osmium tetroxide. The samples were rinsed with distilled water and dehydrated serially in 30, 50, 70, and 90 % ethanol, and three times with 100 % ethanol. After the solvent was replaced with 2-methyl-2-propanol and lyophilized, the samples were placed on carbon adhesive tabs. Each clot was coated with gold and observed using SEM (JSM-6510, JEOL, Tokyo, Japan). Average fiber diameters were measured on micrographs at 10,000 magnification using ImageJ 1.47. The number of branched points was counted in five areas of 4 μm^2 per micrograph [10, 11].

Measurement of thrombin activity

Thrombin activity was measured by monitoring at A_{410} according to the method previously described [5]. DL-BAPA in TBS+Ca^{2+} (5 mM/mL, 40 μL) was preincubated with various sugars in the same buffer (0.2 M, 50 μL) at 37 °C for 5 min in a 96-well microtiter plate. After adding thrombin in the same buffer (15.6 mM, 10 μL) to each well, the plate was mixed and monitored at A_{410} for 210 min with a microplate reader (Vient, DS Pharma Biomedical).

Statistical analysis

Data of experiments were described with mean and standard deviation, and statistical significance was evaluated by a parametric *t* test using Microsoft Excel.

Results

Carbohydrate-binding studies of fibrinogen and fibrin

The specificities of carbohydrate-binding activities of fibrinogen and fibrin were elucidated using sugar-BP probes. As shown in Fig. 1a and b, in both the presence and absence of Ca^{2+}, fibrinogen bound best to α-Man- and α-GalNAc-BP probes. After conversion of fibrinogen to fibrin by thrombin treatment, the overall carbohydrate-binding activities of fibrin were increased, especially to the NeuAc-BP probe in the presence of Ca^{2+} (Fig. 1c). Though Ca^{2+} little affected the carbohydrate-binding activity of fibrinogen, it dramatically suppressed the binding of fibrin to the NeuAc-BP and other sugar-BP probes (Fig. 1d).

Effects of carbohydrates on thrombin-induced fibrin polymerization

Fibrin polymerization was measured by turbidity monitored at A_{350}. Proteolytic processing and polymerization states of fibrin were analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 2a, the turbidity increased time-dependently and reached a plateau in 60 min in the presence of thrombin in TBS+Ca^{2+}, indicating that the polymerization of fibrin was completed. The turbidity increased time-dependently in TBS or TBS containing 5 mM EGTA (TBS+EGTA). In the absence of thrombin, the turbidity remained unchanged irrespective of Ca^{2+} and EGTA concentrations. Figure 2b shows the cross-linking of fibrin that was monitored by SDS-PAGE. The release of fibrinopeptides A and B from Aα and Bβ was observed in the presence and absence of Ca^{2+}. Formation of γ-dimers and α-polymers proceeded time-dependently in the presence of Ca^{2+}, while neither cross-link was formed in the absence of Ca^{2+}. The results in Fig. 2a and b show that the turbidity at A_{350} was consistent with the state of fibrin polymerization and that there was no difference between TBS and TBS+EGTA.

Effects of carbohydrates on thrombin-induced fibrin polymerization were investigated. As shown in Fig. 3a and b, Me α-Man, D-GalNAc, D-GlcNAc and NeuAc markedly inhibited the increase in turbidity due to fibrinogen after adding thrombin. Low concentration of Me α-Man (0.06 mM) inhibited turbidity development in the presence of Ca^{2+} to 64 % compared with control (data not shown). On the other hand, Me β-Glc hardly inhibited the turbidity increase. As shown in Fig. 3c and d, formation of only α-polymers was markedly decreased by D-GalNAc, D-GlcNAc, and NeuAc, suggesting that binding of fibrinogen to an acetyl group is important for inhibition of fibrin cross-linking in the presence of Ca^{2+}. As shown in Fig. 4a and b, in the absence of Ca^{2+}, only Me α-Man inhibited the increase in turbidity due to fibrinogen after adding thrombin. The others did not affect on neither the turbidity nor SDS-PAGE pattern.

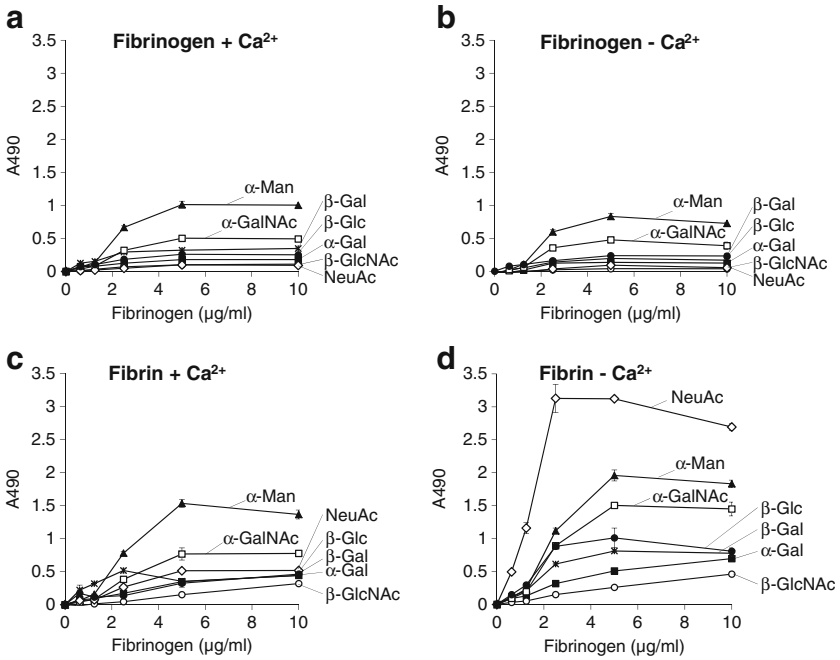


Fig. 1 Carbohydrate-binding activity of fibrinogen and fibrin by ELISA using sugar-BP probes. Fibrinogen was immobilized on 96 well-plate wells and converted to fibrin by thrombin after immobilization on the wells as described in Material and methods. After blocking with skim milk, the immobilized fibrinogen or fibrin was incubated with sugar-BP probes. Sugar-BP probes bound to fibrinogen or fibrin were detected by ELISA using horseradish peroxidase-labeled avidin. **a** Fibrinogen in the presence of 5 mM Ca^{2+}; **b** fibrinogen in the absence of Ca^{2+}; **c** fibrin in the presence of 5 mM Ca^{2+}; **d** fibrin in the absence of Ca^{2+}

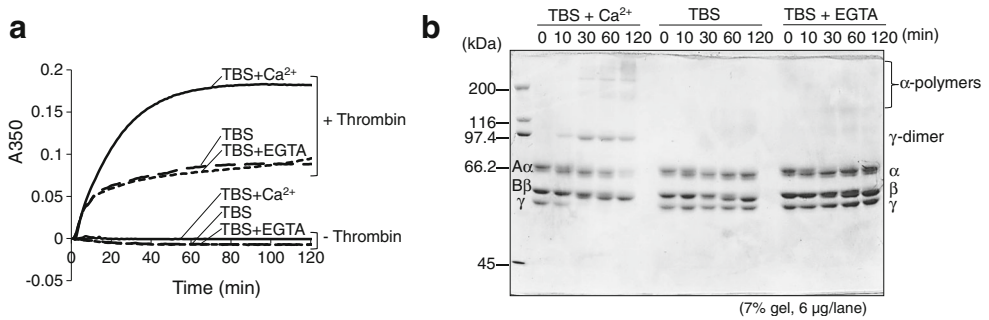


Fig. 2 Effects of thrombin, Ca^{2+}, and EGTA on fibrin polymerization and cross-linking. Fibrinogen (2.2 mg/mL, 90 μL) in TBS in the presence or absence 5 mM $CaCl_2$ or 5 mM EGTA was added to thrombin (0.5 U/mL, 10 μL) in 96-well microtiter plates and plastic tubes. **a** The turbidity of each sample in the 96-well plate was monitored at A_{350} and 37 °C for 120 min. *Solid line* TBS+Ca^{2+}, *broken line*: TBS, *dotted line*: TBS+EGTA. **b** Reaction of samples in plastic tubes was stopped by heating with SDS sample buffer containing 1 % 2-mercaptoethanol at 98 °C for 10 min. The treated samples were subjected to SDS-PAGE and stained with Coomassie brilliant blue R-250

These results show that D-GalNAc, D-GlcNAc, and NeuAc inhibited the thrombin-induced fibrin polymerization that was accompanied by formation of α-polymers in the presence of Ca^{2+}. Me α-Man inhibited the increase in turbidity in both the presence and absence of Ca^{2+}. These results suggest that the inhibition manners of Me α-Man and the acetyl sugars, D-GalNAc, D-GlcNAc and NeuAc, are different.

Effects of carbohydrates on morphological properties of fibrin by SEM

Structures of fibrin clots were observed by SEM. Figure 5 shows the effects of carbohydrates on the clotted fibrin polymer structure. Quantitative assessments of the clot networks in the SEM images are presented in Table. 1. As shown in the SEM images, the fibrin clots of controls contain thick and

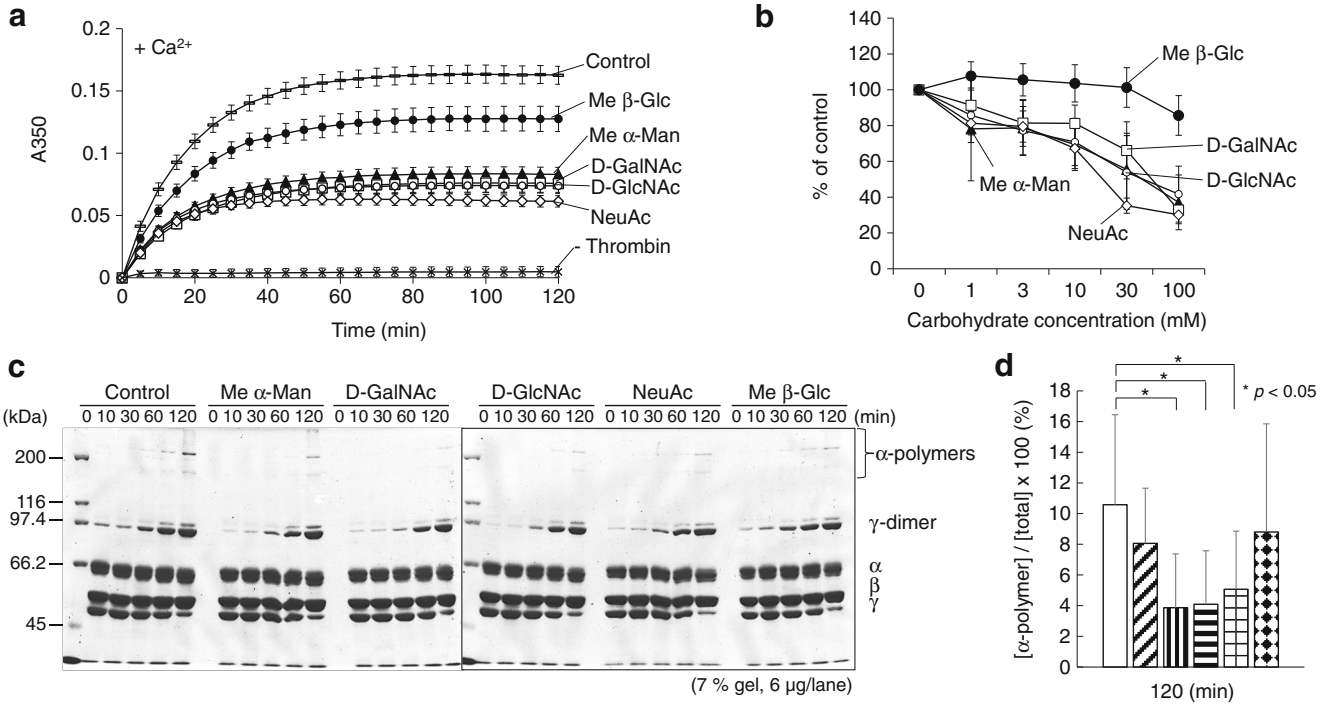


Fig. 3 Effects of carbohydrates on fibrin polymerization and cross-linking in presence of Ca^{2+}. Fibrinogen (5 mg/mL, 40 μL) in TBS+Ca^{2+} was preincubated with various sugars (0.2 M, 50 μL) at 37 °C for 5 min, and thrombin (0.5 U/mL, 10 μL) was added. **a**, **b** The turbidity of each sample in the 96-well plate was monitored at A_{350} and 37 °C for 120 min. Control: —, Me α-Man: *black triangle*, D-GalNAc: *white square*, D-GlcNAc: *white circle*, NeuAc: *white diamond*, Me β-Glc: *black circle*, without thrombin: ×. Time dependency (**a**). Carbohydrate-concentration dependency under the reaction time of 30 min (**b**). **c** Each reaction was stopped, and the sample was subjected to SDS-PAGE and stained with Coomassie brilliant blue R-250. **d** Ratio of α-polymers in Fig. 3c at 120 min were calculated by Image J. Control: □, Me α-Man: ▨, D-GalNAc: ▥, D-GlcNAc: ▤, NeuAc: ⊞, Me β-Glc: ⊡, n=3

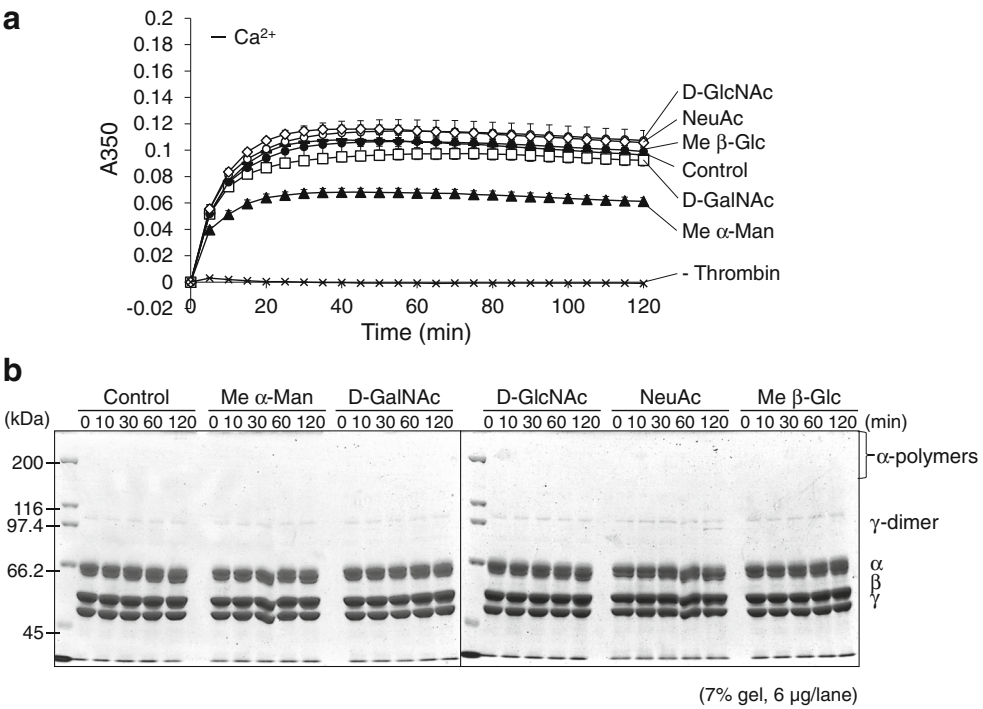


Fig. 4 Effects of carbohydrates on fibrin polymerization and cross-linking in absence of Ca^{2+}. Fibrinogen (5 mg/mL, 40 μL) in TBS was preincubated with various sugars (0.2 M, 50 μL) at 37 °C for 5 min, and thrombin (0.5 U/mL, 10 μL) was added, as in Fig. 3. **a** The time dependently monitoring of turbidity. Control: —, Me α-Man: *black triangle*, D-GalNAc: *white square*, D-GlcNAc: *white circle*, NeuAc: *white diamond*, Me β-Glc: *black circle*, without thrombin: ×. **b** SDS-PAGE and staining with Coomassie brilliant blue R-250

wide porous fibrin fibers. In contrast, thinly branched fibrin strands were formed in the presence of Me α-Man, D-GalNAc, D-GlcNAc, and NeuAc. As shown in Table 1, the diameters of fibrin fibers formed in the presence of the carbohydrates were 42−66 % smaller than those of control fibrin fibers. Furthermore, the numbers of branch points of fibrin fibers formed in the presence of carbohydrates were 240−450 % higher than those of control fibrin fibers.

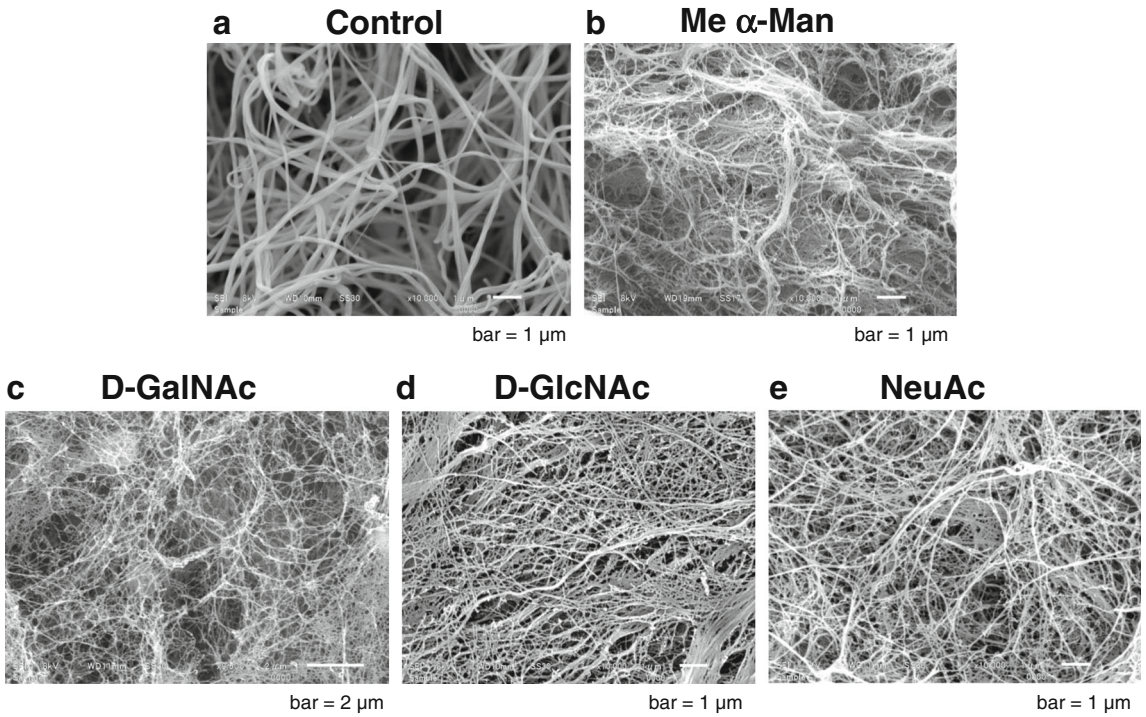


Fig. 5 SEM of fibrin clots formed in the presence of carbohydrates. Fibrinogen (5 mg/mL, 80 μL) was preincubated with various sugars (0.2 M, 100 μL) in TBS+Ca^{2+} and thrombin (0.5 U/mL, 20 μL) was added. **a** Control, **b** Me α-Man, **c** D-GalNAc, **d** D-GlcNAc, **e** NeuAc

Table 1 Morphologic properties of fibrin fibers

	Fiber diameter, nm n=50	No. of branch points/4 μm^2 n=5
Control	82.4±23	36.4±6.3
Me α-Man	34.2±9.3*	93.4±18*
NeuAc	53.4±15*	87.4±9.7*
GalNAc	35.2±12*	133.0±21*
GlcNAc	35.4±7.7*	161.0±30*

*p<0.001

Carbohydrate-binding of thrombin and effects of carbohydrates on thrombin activity

To examine whether thrombin activity is affected by the carbohydrates that inhibited thrombin-induced fibrin polymerization in Figs. 3, 4, and 5, the carbohydrate-binding activity of thrombin was measured using sugar-BP probes in ELISA and the effects of carbohydrates on the peptidolytic activity of thrombin were measured using DL-BAPA as the substrate. As shown in Fig. 6a, thrombin did not show significant carbohydrate-binding activity, in contrast to fibrinogen and fibrin. As shown in Fig. 6b, carbohydrates did not affect the BAPA-hydrolyzing activity. The results together suggest that thrombin does not capture carbohydrates or that the peptidolytic activity of thrombin is not suppressed by carbohydrates.

Discussion

This is the first report that fibrinogen and fibrin have carbohydrate-binding activity (Fig. 1), and that it significantly affects thrombin-induced fibrin polymerization, leading to the formation of finer and more branched fibrin fibers (Figs. 3, 4, and 5 and Table 1). The interference in fibrin polymerization is caused by carbohydrate-binding to fibrin but not to thrombin (Fig. 6).

The fibrinogen monomer is composed of two sets of three polypeptide chains called Aα, Bβ, and γ, as illustrated in Fig. 7. The fibrinogen-fibrin conversion by thrombin consists of three steps: 1) release of fibrinopeptides A (15 to 19 amino acid residues) and B (14 to 21 residues) [12], 2) polymerization of fibrin monomers to form fibrin polymers, and 3) formation of stable fibrin polymers with cross-linking between two adjacent γ chains and between two facing α chains by factor XIII (α-polymers) [13, 14]. The latter two reactions are mainly Ca^{2+}-dependent, but some polymerization of fibrin monomers occurs in step 2 in the absence of Ca^{2+} [15, 16].

Our study revealed the effects of carbohydrate-binding activity and Ca^{2+}on thrombin-induced fibrin polymerization (Fig. 1). The carbohydrate-binding activity of fibrinogen was not affected by Ca^{2+} (Fig. 1a and b). The fibrin converted from fibrinogen by thrombin in the presence of Ca^{2+} (Fig. 1c), which will be a cross-linked fibrin polymer (Fig. 7), bound to carbohydrates more strongly than fibrinogen. The fibrin converted by thrombin in the absence of Ca^{2+} (Fig. 1d), a fibrin monomer (Fig. 7), bound to carbohydrates most strongly, suggesting that the main carbohydrate-recognition site of fibrin may be located in a region of exposed knobs and partial dissociation of the αC regions [14]. The binding activity of fibrin to the NeuAc-BP probe markedly increased in the absence of Ca^{2+} (Fig. 1d). This increase will be due to a change in the charge of the fibrin monomer induced by releasing negatively-charged fibrinopeptides A and B.

Development of turbidity by thrombin in the presence of Ca^{2+} demonstrates the formation of cross-linked fibrin

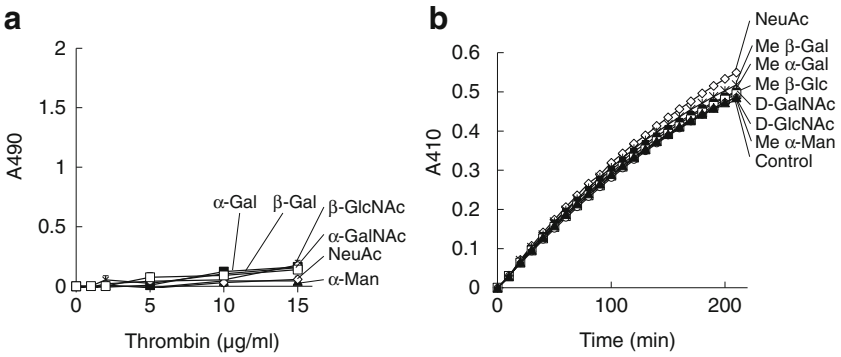


Fig. 6 Carbohydrate-binding activity of thrombin and effects of carbohydrates on thrombin activity. **a** Carbohydrate-binding activity: thrombin was immobilized on 96-well plates and incubated with sugar-BP probes. The sugar-BP probes bound to thrombin were detected by horseradish peroxidase-labeled avidin as described in Fig. 1a. **b** Thrombin activity: reaction mixtures comprised of thrombin (15.6 mM, 10 μL), DL-BAPA (5 mM/mL, 40 μL), and various sugars (0.2 M, 50 μL) in TBS+Ca^{2+} in a 96-well microtiter plate was monitored at A_{410} and 37 °C for 210 min

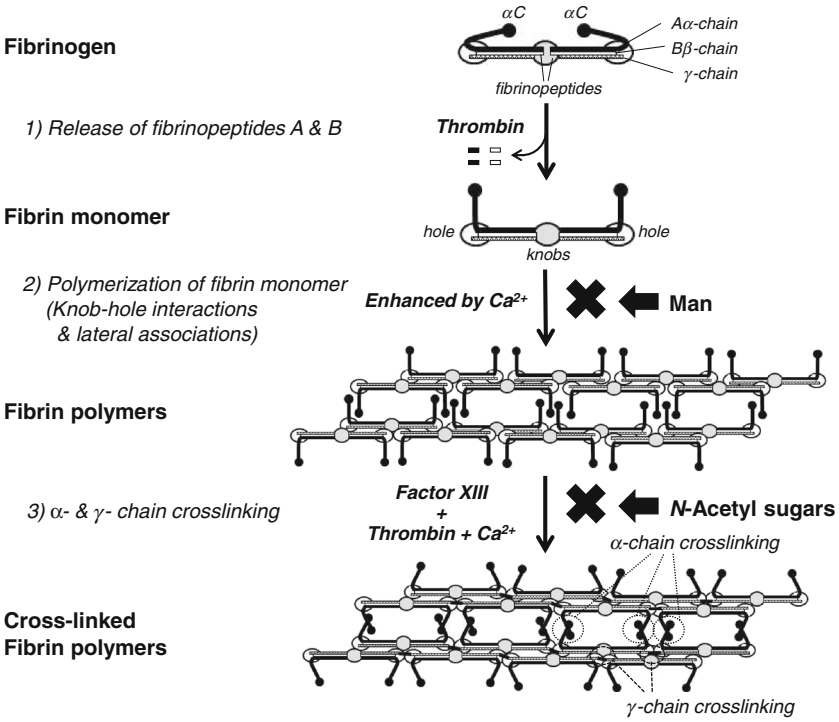


Fig. 7 Schematic illustration of inhibition mechanism on fibrin clotting by carbohydrates. Man and *N*-acetylated sugars inhibited different stages of fibrin clotting. Man inhibited Ca^{2+}-independent fibrin polymerization in step 2. On the other hand, *N*-acetylated sugars inhibited the last step, stabilization by α-chain crosslinking

polymers detected as α polymers and γ dimers, while that in the absence of Ca^{2+} shows formation of fibrin polymers without detectable α polymers and γ dimers (Fig. 2). The binding of Me α-Man to fibrinogen inhibited the increase of turbidity of fibrin in the presence and absence of Ca^{2+} (Fig. 3a and Fig. 4a), and fibrin fibers formed thin and branched fibers in the presence of Me α-Man (Fig. 5b), suggesting that Me α-Man inhibited step 2 and will decrease the subsequent step 3 (Fig. 7). On the other hand, the binding of *N*-acetyl sugars to fibrinogen inhibited turbidity development of fibrin only in the presence of Ca^{2+} (Fig. 3a) but not in its absence (Fig. 4a), and fibrin fibers became thin and branched (Fig. 5c–e) as in the presence of Me α-Man. The results suggest that *N*-acetyl sugars inhibited step 3 (Fig. 7). The roughness of the curves in the presence of sugars (Fig. 3b) may suggest that the fibrin polymerization during the process may be affected by various sugars at multiple steps, although the precise reaction mechanism cannot be revealed by this assay.

Blood concentrations of Man and NeuAc are approximately 0.02–0.055 mM [17, 18] and 1.6–2.2 mM [19], respectively. Me α-Man and NeuAc inhibited turbidity development at fibrinogen-fibrin conversion at nearly physiological concentrations in this study.

It had been reported that fibrinogen binds to heparin and hyaluronan [2, 3]. The anticoagulant action of heparin lies in its ability to bind to and enhance the inhibitory activity of an anti-thrombin against several serine proteases of the coagulation system [20]. The carbohydrate-binding activity of fibrinogen found in this study is distinct from the heparin-binding because these carbohydrates do not bind to thrombin. Heparin did not exhibit anticoagulant activity, decreasing development of fibrin clots due to turbidity and fibrin polymers under the same conditions as this study (data not shown). On the other hand, ^{125}I-fibrinogen bound to hyaluronan-Sepharose, which can be displaced by free hyaluronan but not by either of the monosaccharide components of this polymer, glucuronic acid, and GlcNAc [3]. Hyaluronan enhanced fibrin formation [21], which has opposite effects of the carbohydrate-binding activity of fibrinogen in this study.

This report reveals that fibrinogen and fibrin have carbohydrate-binding activities that significantly affect coagulation activity. The anticoagulation activity of carbohydrates has a different mechanism from that of heparin. The surfaces of exogenous microorganisms, blood cells, and platelets are covered with carbohydrates [22]. Therefore, the carbohydrate-binding activities of fibrinogen and fibrin may have the functions attaching blood clots to the surface of blood vessel endothelium for hemostasis.

In addition, a high concentration of carbohydrates such as NeuAc in the body is a strong predictor of malignancy, heart disease, and diabetes that is beyond the effect of established risk factors [19, 23, 24]. The results in this study will contribute to understanding of the blood anti-coagulation mechanism of disease with increasing NeuAc concentration.

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