Versican G3 Domain Promotes Blood Coagulation through Suppressing the Activity of Tissue Factor Pathway Inhibitor-1*

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We have detected versican, a member of the large chondroitin sulfate proteoglycans, and its degraded C-terminal G3 fragments in human plasma and observed that the versican G3 domain promoted blood coagulation. Silencing G3 expression with small interfering RNA reduced the effect of G3 on coagulation. Plasma coagulation assays suggest that G3 enhances coagulation irrespective of its actions on platelets and white blood cells. To examine how versican affected blood coagulation, we used normal human plasma and different types of coagulation factor-deficient plasmas. The experiments indicated that versican enhanced coagulation through the extrinsic pathway, and that Factor VII was the target molecule. FVII activity assays showed that G3 activated FVII in the presence of plasma but not with purified FVII directly. Yeast two-hybrid, immunoprecipitation, and gel co-migration assays showed that G3 interacted with the tissue factor pathway inhibitor-1 (TFPI-1). TFPI-1 activity assays suggested that G3 inhibited TFPI-1 activity, allowing FVIIa and FXa to facilitate the coagulation process. G3-induced blood coagulation was further confirmed with a mouse model in a real-time manner. Taken together, these results indicate that versican may represent a new target for the development of therapies against atherosclerosis.

Blood coagulation is a host defense system that maintains the integrity of the high pressure blood circulatory system. To prevent excessive blood loss after tissue injury, components in the system, including vascular endothelial cells, platelets, and plasma coagulation proteins, are mobilized for blood coagulation. The extracellular matrix (ECM) molecules play an important role in blood coagulation. Versican is a major extracellular chondroitin sulfate proteoglycan detected in the vessel wall, where it contributes to the formation of blood vessels. It is highly expressed by aortic endothelial cells (1) and vascular smooth muscle cells (2).

As a member of the large aggregating chondroitin sulfate proteoglycan family, versican is made up of an N-terminal G1 domain, a glycosaminoglycan (GAG) attachment region, and a C terminus containing a selectin-like (or G3) domain. The G1 domain is composed of an immunoglobulin-like motif and two proteoglycan tandem repeats (3). The G3 domain contains two epidermal growth factor-like repeats, a selectin-like motif (also known as carbohydrate recognition domain), and a complement binding protein-like motif (3).

In vascular diseases, versican expression and accumulation increase after human cardiac allograft and coronary angioplasty (4). Increased expression is evident in the thickened myxoid intimas, in the loose ECM of restenotic lesions, in the atherosclerotic plaque, and in the thrombus interface (5, 6). In advancing atherosclerotic lesions, decreased sulfation of versican GAG chains may predispose the lesions to thrombosis by disrupting osmotic regulation, limiting avidity for antithrombin (7). Furthermore, metalloproteinases, ADAMTS (A Disintegrin And Metalloprotease with Thrombospondin repeats), and plasmin, which may become activated in atherosclerotic lesions or abnormal conditions, are known to cleave versican and other matrix molecules, resulting in the release of versican fragments into the plasma (8–10). We have recently demonstrated that G3-containing fragments are highly present in plasma and that the G3 domain interacts with P-selectin glycoprotein ligand-1 and induces leukocyte aggregation (11). This study was designed to investigate the effect of versican in blood coagulation.

EXPERIMENTAL PROCEDURES

Materials —Plasma and coagulation factor-depleted plasma were purchased from Precision Biologic Inc. (Dartmouth, Canada) and were routinely used in the Laboratory of Biochemistry at Sunnybrook & Women’s College Health Sciences Centre. P-Pack was purchased from Calbiochem (Stanford, CT). CAOSERT FVII assay kits and phospholipid were purchased from Chromogenix Instrumentation Laboratory SpA (Milan, Italy). ACTICROME TFPI activity assay kits, mouse anti-human TFPI monoclonal antibody, and rabbit anti-human TFPI polyclonal antibody were purchased from American Diagnostica Inc. (Stanford, CT). Anti-tissue factor antibody (CD142, clone number HTF-1) was purchased from BD Biosciences. ECL Western blot detection kit was from Amersham Biosciences. Antibodies against human versican, horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody, oligonucleotides, and all of the chemicals were from Sigma.

Coagulation Assays —In whole blood coagulation assays, 1 ml of culture medium from G3- or vector-transfected U87 cells was added into plastic tubes and prewarmed at 37 °C in a water bath, to which 1 ml of freshly withdrawn blood was added and mixed gently. The mixture was then incubated at 37 °C. Blood coagulation was monitored every 30 s. Once blood clots formed, the time required for coagulation was recorded. In plasma coagulation assays, sodium citrate (225 µl, 3.2%) was mixed with 225 µl of platelet-rich plasma (PRP) or platelet poor plasma (PPP), to which an equal volume (450 µl) of culture medium from G3- or vector-transfected cells was added. The mixture was incu-
bated at 37 °C for 5 min in a water bath. CaCl₂ (50 μL, 0.12 mM) was added to each tube and gently shaken. The mixture was incubated at 37 °C, and plasma coagulation was monitored every 30 s. The reaction was stopped once plasma clots formed.

Platelet Aggregation Assay — P-Pack (100 μM), a chloromethylketone that is an anti-coagulant inhibiting α-thrombin through a non-calcium pathway, or sodium citrate (3.2%) was added to freshly withdrawn human blood. Platelet-rich plasma was prepared. Culture medium from vector- or G3-transfected U87 cells was mixed with P-Pack or sodium citrate-treated anti-coagulated plasma in a 1:1 ratio. The mixture was warmed up in a platelet aggregation chromogenic kinetic system (Helena Laboratories, Beaumont, TX) for 3 min. CaCl₂ (50 μL, 0.12 mM) was added to activate platelet aggregation. The instrument automatically recorded the aggregation activity.

Yeast Two-hybrid Assays — We used the Gal4 yeast two-hybrid system from Clontech to identify the domains of TFPI that interacted with the versican G3 domain. The G3 domain was inserted into the plasmid pGBDC1 to generate the construct pGBDC1-G3 flanked by BamHI and SalI sites.

Seven recombinant constructs of TFPI were generated in the yeast activation vector pGADT7. Each fragment was amplified by PCR, digested with EcoRI and BamHI, and inserted into EcoRI- and BamHI-digested pGADT7. Two primers, TFPI29NEcoRI (5′-cccgaattctgtgcattcaaggcggatgat) and TFPI124CBamHI (5′-cccccatgcgtgactctggtctgtg), were used to produce the construct TFPI-1; primers TFPI54NEcoRI (5′-ccggattctgctttttggaagaagatcct) and TFPI124CBamHI (5′-ccggatccgaaatctggcttttcttg-3g), were used to produce the construct TFPI-2; primers TFPI54NEcoRI and TFPI216CBamHI (5′-ccggatccgaaatctggcttttcttg-3g) produced TFPI-3; primers TFPI54NEcoRI and TFPI304CBamHI (5′-ccggatccgaaatctggcttttcttg-3g) produced TFPI-4; primers TFPI125NEcoRI (5′-ccggatccgaaatctggcttttcttg-3g) and TFPI216CBamHI produced TFPI-5; primers TFPI125NEcoRI and TFPI304CBamHI produced TFPI-6; and primers TFPI217NEcoRI (5′-ccggatccgaaatctggcttttcttg-3g) and TFPI304CBamHI produced TFPI-7. Sequencing of positive clones was performed by Inno Biotech (Toronto, Canada). Yeast strain JP-69–4A was co-transfected with yeast constructs was carried out by Western blot, flow cytometry, and immunofluorescence staining as described previously (14, 15). Immunoprecipitation was also performed as previously described (16).

Construct Generation and Expression — To generate an siRNA construct targeting the G3 domain, a target sequence (nucleotides 10789–10808, cactcatcactcgctgtac) of chicken versican (GenBank accession number D13542) was inserted into the pSuper plasmid according to the manufacturer’s instructions (12). The identity of the target sequence was verified by DNA sequencing. The silencing effect of the siRNA construct on G3 expression was analyzed by Western blot. We have previously used this technique to effectively silence versican expression (13).

The astrocytoma cell line U87 was transfected with recombinant constructs using Lipofectamine. Expression of the recombinant constructs was carried out by Western blot, flow cytometry, and immunofluorescence staining as described previously (14, 15). Immunoprecipitation was also performed as previously described (16).

COASET FVII Assays — The COASET FVII activity assay is based on a two-stage reaction. First, FX is activated to FXa via the extrinsic pathway (FVII-thromboplastin). FVII is completely converted to FVIIa during this process. The activated Factor Xa (or FXa) hydrolyzes the chromogenic substrate S-2765, which liberates the chromophoric group p-nitroaniline. The intensity of color and the amount of FXa is therefore proportional to FVII activity. Different components were added into the reaction system according to the instructions in the manufacturer’s instructions.

Briefly, for the plasma assay, 20 μl of 1:10 diluted thromboplastin, 20 μl of purified G3, 20 μl of control protein, or 20 μl of buffer (as blank) were mixed with 120 μl of commercial FX (0.5 units/ml) and 60 μl of CaCl₂ (40 mmol/liter). A blank control (Tris-bovine serum albumin working buffer) was included in each series. 200 μl of FII-deficient plasma (diluted 10-fold) was added into the mixture and incubated at 37°C for 7 min. Substrate S-2765 (200 μl) was added and incubated at 37°C for 5 min. Finally, 20% acetic acid (200 μl) was added to stop the reaction. The absorbance was measured within 4 h in a photometer at a wavelength of 405 nm for the relative activity of FVII, zeroed against the blank control.

TFPI Activity Assays — ACTICHROME TFPI activity assay kits were used to measure the ability of TFPI to inhibit the catalytic activity of the TF-FVIIa complex to activate FX to FXa. After incubation of the test samples with TF-FVIIa and FX, the residual activity of the TF-FVII complex was measured using a highly specific chromogenic substrate, SPECTROZYME, which releases the chromophore p-nitroaniline upon specific cleavage by FXa. A standard activity curve was generated according to the manufacturer’s instructions in each assay. Briefly, TFPI (20 μl) at different concentrations were added for each test. TF-FVIIa (20 μl) was mixed with TFPI and incubated at 37°C for 30 min. Human FX (20 μl) was added to the mixture and incubated at 37°C for 15 min. EDTA (20 μl) and then SPECTROZYME FXa substrate (20 μl) was added to each well to determine the absorbance of the solution at 405 nm every 5 min. The reaction was stopped at 25 min by adding glacial acetic acid (50 μl) to each well.

For the plasma TFPI activity assay, 1:20 diluted human TFPI reference plasma (10 μl) was mixed with fresh medium (10 μl) or medium from vector- or G3-transfected cells. In a separate experiment, purified G3 (10 μl) or purified mini-versican dissolved in 50 mM Tris-Cl, pH 8.0, or buffer alone (10 μl, negative control) was used. For the commercial TFPI activity assay, 100 unit/ml TFPI (10 μl) was mixed with an equal volume of fresh medium or medium from vector- or G3-transfected cells. In a separate experiment, purified G3 protein (10 μl) or purified mini-versican dissolved in 50 mM Tris-Cl, pH 8.0, or buffer alone (10 μl) was used. The experimental procedures were as described for the standard TFPI activity assay. The absorbance of p-nitroaniline was measured at 405 nm. The value was compared with those obtained from the standard curve.

For antibody blocking experiments, medium from vector- or G3-transfected cells (10 μl) was mixed with a polyclonal antibody against G3 (5 μl) raised by our lab previously (17) or mixed with rabbit serum (5 μl) as a negative control. The mixture was incubated at 4°C for 2 h. The serum-containing samples were used in the assay as above, in which only 5 μl of 200 unit/ml TFPI were added to bring each well to the same volume and same TFPI activity.

In Vivo Coagulation Model — We have recently established a mouse model to examine blood coagulation in vivo in a real-time manner. To examine the effect of versican G3 product on blood coagulation, we introduced G3-containing conditioned medium or purified G3 product into CD-1 mice (Charles River) intraperitoneally. Thirty minutes after the injection, the mouse was anesthetized. Blood circulation in the edges of the liver or in the intestines was observed under an inverted light microscope and photographed. In another set of experiments, sodium thioglycollate, which causes damage to the endothelium and extravasation of blood, was introduced into the mice (3%, 1–2 ml/mouse) intraperitoneally 30 min following G3 injection. The mouse was anesthetized 30 min after the injection of sodium thioglycollate, followed by examination of blood coagulation in the intestines. Animal
experiments were performed following the guidelines of the Sunnybrook Animal Committee.

RESULTS

Versican G3 Enhances Coagulation—We have previously demonstrated that versican G3-containing fragments are present in plasma and that G3 induces leukocyte aggregation (11). In this study, we tested whether versican G3 product induced blood coagulation. Medium containing G3 (Fig. 1A) was added to freshly withdrawn human blood in plastic tubes. The addition of G3-containing medium promoted whole blood coagulation as compared with culture medium from vector-transfected cells (Fig. 1B). G3-containing medium was then diluted, and we observed that plasma coagulation was induced by G3-containing medium in a concentration-dependent manner (not shown). G3 was next purified using nickel-nitrilotriacetic acid affinity columns. The purified product was confirmed by Western blot probed with the monoclonal antibody 4B6, which recognizes an epitope in the leading peptide of the G3 product (Fig. 1A). The smaller band in the lysate is the unglycosylated G3 peptide, as G3 contains potential sites for glycosylation. The addition of purified G3 to plasma induced coagulation, indicating that leukocytes are not required for coagulation (Fig. 1C). The effects of G3-containing medium (Fig. 1D) and purified G3 (Fig. 1E) on coagulation were concentration-dependent.

To further validate the direct involvement of G3 in coagulation, we used small interfering RNA to reduce G3 expression. One construct (siRNA-ver10789) containing a target sequence against the G3 domain was found to greatly silence expression of the G3 construct in COS-7 cells (Fig. 2A). G3-expressing U87 cells (stable cell line) were co-transfected with siRNA-ver10789 and pcDNA3.1/Hygro or pSuper and pcDNA3.1/Hygro. Cell lines stably transfected with siRNA-ver10789 or pSuper were selected with hygromycin. Expression of G3 in the selected cell lines was analyzed by Western blotting on hygromycin-resistant cell lines. The addition of G3-containing medium promoted whole blood coagulation (Fig. 2B). In coagulation experiments, culture medium from siRNA-ver10789-transfected COS-7 cells (Fig. 2C) and U87 cells (Fig. 2D) caused reduced activity in coagulation as compared with the medium from G3-transfected cells. These results indicate that the G3 product was responsible for the enhanced coagulation.

Because plasma contains large numbers of platelets, we tested whether platelets play a role in G3-induced blood coagulation. Conditioned culture medium from G3 and vector-transfected U87 cells were mixed with PRP or PPP. The mixture was added to human plasma, and plasma coagulation was monitored. The addition of G3-containing medium promoted plasma coagulation (Fig. 3A). When mixed with either G3-containing medium or medium from vector-transfected cells, PRP produced more rapid coagulation than did PPP. This result implies that platelets are important in plasma coagulation; the platelet surface may release some coagulation-enhancing factors after activation. To further confirm this result, conditioned medium from G3- and vector-
transfected cells was used to dilute PRP to produce plasma containing different concentrations of platelets. Again, G3-containing medium stimulated plasma coagulation (Fig. 3B). Although higher numbers of platelets also enhanced coagulation under both G3 and control conditions, it appeared that G3 induced coagulation of both PPP and PRP. To test whether G3-containing medium could affect platelet aggregation, culture medium from vector- or G3-transfected cells were mixed with platelets in the presence of P-Pack or sodium citrate. Little difference was observed between the media from G3- and vector-transfected cells (Fig. 3C).

Involvement of FVII in G3-enhanced Coagulation—Because coagulation factors play crucial roles in blood and plasma coagulation, we tested different coagulation factor(s) to find which was involved in G3-induced coagulation. Medium from G3- or vector-transfected cells was added to plasma depleted of different individual coagulation factors. The experiments indicated that the effect of G3 on plasma coagulation was abolished when the plasma was depleted of FII, FVII, or FX (Fig. 4A). Our results indicated that plasma depleted of coagulation factors of the intrinsic pathway (FXII, FXI, FIX, and FVIII) could coagulate, but plasma depleted of FVII located in the extrinsic pathway, or FII or FX (both located in the common pathway), could no longer coagulate. This suggested that G3 might function by modulating FVII activity. To confirm this, different proportions of normal plasma were added to FVII-deficient plasma, followed by incubation with culture medium from G3- or vector-transfected cells. The addition of normal plasma restored the effect of G3 on plasma coagulation, presumably through the addition of FVII to the mixture (Fig. 4B). However, it is not known why the effect of G3 was not abolished by FV depletion or why G3 did not enhance coagulation of FVIII-depleted plasma.

In the extrinsic system, tissue factor forms a complex with calcium ions and FVII. FVII is normally inactive but becomes activated (FVIIa) by binding to TF. The TF-FVIIa complex acts on other coagulation factors and launches the extrinsic pathway, culminating in the generation of thrombin and the formation of a fibrin clot (18). The FVIIa-like activities of culture medium from G3- or vector-transfected cells were tested using the COASET FVII Assay kits. Thromboplastin containing TF was used as a positive control to activate FVII. G3 exhibited TF-like activity in the system containing plasma (activating FVII to FVIIa), but the medium from vector-transfected cells did not (Fig. 5A). When the plasma was replaced with phospholipids and incubated with either thromboplastin, FVIIa, FVII, or medium from G3- or vector-transfected cells, neither medium exhibited FVII-like activity (Fig. 5B). This suggested that G3 could not activate FVII and that the G3-containing medium did not contain TF. The latter was confirmed by Western blot probed with anti-tissue factor antibody (Fig. 5C).

G3 Functions by Suppressing the Inhibitory Effect of TFPI-1 on Coagulation— TFPI-1 inhibits tissue factor procoagulant activity and
the activity of FXa by directly binding to FXa via its Kunitz domain 2. It also interacts with the TF-FVIIa complex via its Kunitz domain 1, forming an inactive quaternary complex. We tested whether versican G3 interacted with TFPI-1 and modulated its coagulation activity. Recombinant constructs containing different domains of TFPI-1 were generated in the yeast expression vector pGADT7 for yeast two-hybrid assays (Fig. 6A). The experiments indicated that constructs containing both Kunitz domains 1 and 2 interacted with the G3 domain (Fig. 6B). However, constructs containing only one of these domains did not interact with the G3 domain. This suggested that the interaction of TFPI-1 with the G3 domain required both Kunitz domains 1 and 2.

The interaction of versican G3 with TFPI-1 was confirmed using human plasma. Human plasma was first subjected to 0.7% agarose gel electrophoresis, followed by immunoblotting probed with 2B1 antibody (Fig. 6D). Human plasma was also immunoprecipitated with anti-G3 polyclonal antibody (Fig. 6E). In another co-IP assay, anti-TFPI-1 antibody was used to pull-down TFPI-1, followed by Western blot detecting G3 protein. IP, immunoprecipitation.

FIGURE 6. G3 binds TFPI-1. A, amino acid structure of TFPI-1 showing motif structures. Recombinant constructs containing different domains of TFPI-1 were generated in the yeast expression vector pGADT7 for yeast two-hybrid assays. B, yeast two-hybrid binding assays of TFPI-1 constructs with the G3 domain. Constructs containing both FVIIa- and FXa-binding domains of TFPI-1 interacted with the G3 domain (n = 3; **, p < 0.01). C, human plasma was subjected to agarose gel electrophoresis, followed by immunoblotting probed with 2B1 or anti-TFPI-1 antibodies. D, human plasma was incubated with rabbit anti-G3 polyclonal antibody (lane 1), beads alone (lane 2), or rabbit anti-human endostatin (lane 3), followed by incubation with protein G beads. After washing, proteins binding to the beads were subjected to Western blot probed with an anti-TFPI-1 antibody. Human plasma (diluted 8-fold) was used as a positive control (lane 4). TFPI-1 co-precipitated with G3. E, in another co-IP assay, anti-TFPI-1 antibody was used to pull-down TFPI-1, followed by Western blot detecting G3 protein. IP, immunoprecipitation.
G3 Induces Coagulation

FIGURE 7. Addition of purified G3 product abolishes the effect of TFPI-1 on plasma coagulation. A, to measure TFPI-1 activity, a standard activity curve was generated according to the manufacturer’s instructions. B, fresh medium, culture medium from G3- or vector-transfected cells (10 μl), purified mini-versican (p-versican, 10 μl, 1 μg/ml), and purified G3 (p-G3, 10 μl, 1 μg/ml) were tested. Both versican and G3 suppressed the function of TFPI-1, increasing FXa activity. C, in the same assay system, human plasma was replaced with purified TFPI-1. Both versican and G3 suppressed the function of TFPI-1, leading to greater FXa activity. D, G3 and mini-versican products were preincubated with anti-G3 polyclonal antibody or normal serum as a control. The TFPI inhibitory activity of G3 and versican was suppressed. E, to examine the effect of endogenous TFPI-1 on plasma coagulation, purified TFPI-1 was not added to the assay system. Anti-G3 antibody reduced plasma coagulation, F, to test the role of endogenous G3-containing fragments in plasma coagulation. G3 antibody was added to the assay system, resulting in a reduction in plasma coagulation (n = 3; *, p < 0.02; **, p < 0.01).

in a reduction of plasma coagulation (Fig. 7F). We conclude that versican and versican G3 products can induce coagulation by modulating TFPI activity.

**G3 Induces Blood Coagulation in Vivo**—We tested the role of G3 in blood coagulation in vivo. Conditioned medium from U87 cells stably transfected with the G3 construct or a control vector was intraperitoneally injected into mice. G3-induced blood coagulation was detected in the small blood vessels at the edges of the liver where the tissue was thin enough to allow light transmission (Fig. 8A). This was also found in the intestines (Fig. 8B). All of the animals (four in each group) exhibited similar coagulation results. The mice were also treated with sodium thioglycollate, a chemical causing damage to the endothelium resulting in bleeding and extravasation. Treated animals had 5–10-fold increases in white blood cell numbers in the acites compared with controls (data not shown). In the presence of G3, extensive coagulation was detected (Fig. 8C). In some areas, a whole vessel was completely blocked, whereas an adjacent vessel was circulating well (data not shown). As a result, mice survived longer than the controls, because G3-induced coagulation prevented extensive loss of blood (data not shown). Purified G3 product was also introduced into the mice, and similar results were obtained (Fig. 8D). These experiments thus provided direct evidence that G3 could induce blood coagulation in vivo.

**DISCUSSION**

Versican is the major chondroitin sulfate proteoglycan in blood vessel walls, where it contributes to the formation of new vessels. In vitro studies have shown that versican modulates arterial and vascular smooth muscle cell adhesion, migration, and proliferation (19). Versican expression and accumulation increase after cardiac allograft and coronary angioplasty (4, 20). The increase is even more evident in the thickened myxoid intimas, atherosclerotic plaque, and loose ECM of restenotic lesions (5, 21). It is also evident in the intimal layer of normal arteries and in the intima formed after arterial injury, as compared with the underlying media (22). Increased versican expression is consistently associated with the development of vascular diseases. It is not known, however, whether it is the cause of disease development or a consequence of disease. Our observation that versican can induce blood coagulation suggests that versican can induce thrombosis, especially in the advanced stages of vascular diseases.

Versican is also released into the circulatory system through degradation. Aneurysmal tissue shows peripheral infiltrates of inflammatory cells (23), and these cells, in particular the macrophages, produce matrix metalloproteinases that cleave matrix molecules including versican. It is conceivable that G3-containing fragments, important components in the induction of blood coagulation through binding and suppressing the function of TFPI-1, are also released in this process. Some of this loss could be due to selective degradation of versican, because versican-degrading enzymes increase during aneurysmal expansion (24). We have also demonstrated that human plasma contains versican and G3-containing fragments, which induce leukocyte aggregation. Removal of these versican fragments inhibits these processes (11). In advancing atherosclerotic lesions, decreased sulfation of versican GAG chains may promote thrombosis by disrupting osmotic regulation (7). It is not known whether the GAG chains affect TFPI action. Nevertheless, it would not be surprising if multiple mechanisms were found to be involved in versican-associated coagulation, because circulating versican could produce multiple effects on the system.

In the extracellular pool, versican is known to associate with a number of molecules (25). Versican may play an important role in hemostasis by binding to the ECM molecules and forming high molecular weight hydrophilic complexes. During tissue injury, induction of blood coagulation could allow versican to stop bleeding and prevent blood loss, an alternative mechanism to GPIb in regulating bleeding (26). In advanced atherosclerosis, disruption of osmotic regulation caused by decreased sulfation of GAG chains in the versican CS domain may predispose to thrombosis (7).

It has been reported that fibronectin co-precipitates with versican (27). Recently, we have identified the C-terminal G3 domain of versican...
as the fibronectin-binding motif (16). We have also shown that the G3 domain, fibronectin, and vascular endothelial growth factor (VEGF) form a complex, and others have shown that fibronectin modulates endothelial cell activities through its binding with VEGF (28). Because fibronectin also binds to many other ECM molecules, it is conceivable that versican could facilitate blood coagulation by forming complexes with these ECM molecules through binding to fibronectin.

Versican and other proteoglycans have been found to be deposited with lipoproteins in human atherosclerotic lesions, and large chondroitin sulfate proteoglycan-lipoprotein complexes have been isolated from the lesions (29). Studies have also indicated that lipoprotein binds chondroitin sulfate proteoglycans through their GAG chains (30).

The treatment of the cultures with oxidized lipoprotein and transforming growth factor-β enhances binding of versican to lipoproteins (31). Because lipoproteins are a major component in blood and exert a central role in thrombosis, the interaction of versican with lipoproteins could significantly contribute to these processes.

Recently, it was reported that the interaction of PSGL-1 with selectins enhances the recruitment of leukocytes and leukocyte microparticles to thrombi (32). The accumulation of circulating microparticles to thrombi is important for normal tissue factor accumulation and fibrin generation in thrombi. Platelets are recruited to the injured site and activate blood coagulation through the interaction of PSGL-1 and P-selectin (32). We also reported that G3 forms complexes (33) and induces leukocyte aggregation (11). This may be another mechanism by which G3 induces blood coagulation and may explain how G3 could enhance coagulation in FV-depleted plasma. This appears to be unique to G3 because it possesses multiple mechanisms associated with blood coagulation.

Our report that versican interacts with TFPI-1 has added a new mechanism to the understanding of the effect of versican in blood coagulation. Our results suggest that versican and versican G3 products modulate TFPI activity by binding to the Kunitz domains 1 and 2. After the G3 domain binds to the FVIIa- and FXa-binding domains of TFPI, TFPI releases FVIIa and FXa. The released FVIIa can also activate FX to generate more FXa. The activated FXa can then activate FII to produce FIIa, which induces the formation of fibrin clots or converts SPEC-TROZYME to yellow p-nitroaniline in our assays. The interaction of the versican G3 domain with TFPI-1 thus represents a different pathway in blood coagulation. Yet unresolved is the question of whether circulating levels of versican are high enough to trigger this effect; however, if G3-containing fragments are extensively released to the circulation, it is likely that blood coagulation could be induced. This is supported by our real-time coagulation experiments in a mouse model, since G3 induced blood coagulation when it was injected intraperitoneally. Its effect on blood coagulation also reduced the extensive loss of blood when sodium thioglycollate was introduced into the animals.

In summary, we have demonstrated that the G3 domain of versican induces blood and plasma coagulation. Versican enhances coagulation through the extrinsic pathway, and Factor VII was the target molecule. Further, we demonstrated that the G3 domain interacted with TFPI-1. Our findings suggest that versican enhances blood coagulation through binding to TFPI-1 and suppressing its inhibitory role in blood coagulation. Accumulating evidence suggests that versican is a key molecule in atherosclerosis, and our results have illustrated an exciting new aspect in the genesis of this disease. Future directions will involve the development of molecular genetic models to inhibit the role of versican in this disease.

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