The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis

Katrin Faye Nickel, Göran Ronquist, Florian Langer, Linda Labberton, Tobias A. Fuchs, Carsten Bokemeyer, Guido Sauter, Markus Graefen, Nigel Mackman, Evi X. Stavrou, Gunnar Ronquist, Thomas Renné

From the:
(1) Institute of Clinical Chemistry and Laboratory Medicine; (5) Clinical Department of Hematology and Oncology, Center for Oncology; (6) Institute for Pathology; (7) Martini-Clinic, Prostate Cancer Center, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.
(2) Division of Clinical Chemistry, Department of Molecular Medicine and Surgery, (3) Center of Molecular Medicine, Karolinska Institutet, Stockholm, Sweden.
(4) Clinical Chemistry, Department of Medical Sciences, Clinical Chemistry, University Hospital of Uppsala, Uppsala, Sweden.
(8) Division of Hematology/Oncology, Department of Medicine, University of North Carolina, Chapel Hill, USA.
(9) Divisions of Hematology and Oncology, Department of Medicine, Case Western Reserve University, Cleveland, OH; Department of Medicine, Louis Stokes Veterans Administration Hospital, Cleveland, OH, USA.

*Author for correspondence:
Thomas Renné, M.D. Ph.D.
Clinical Chemistry, Department of Molecular Medicine and Surgery
Karolinska Institutet
Karolinska University Hospital Solna (L1:00)
SE-171 76 Stockholm, Sweden
and
Institute of Clinical Chemistry and Laboratory Medicine (O26)
University Medical Center Hamburg-Eppendorf
D-20246 Hamburg, Germany
Tel.: +46-8-517-73390; +49-40-7410 58984
Fax: +46-310376; +49-40-7410 57576
Email: thomas@renne.net

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2. Targeting the polyphosphate/factor XII pathway reduces procoagulant activity in prostate cancer patient plasma and may permit safe anticoagulation.

ABSTRACT

Cancer is a leading cause of thrombosis. We identify a new procoagulant mechanism that contributes to thromboembolism in prostate cancer and allows for safe anticoagulation therapy development.

Prostate cancer-mediated procoagulant activity was reduced in plasma in the absence of factor XII or its substrate of the intrinsic coagulation pathway, factor XI. Prostate cancer cells and secreted prostasomes expose long chain polyphosphate on their surface that co-localized with active factor XII and initiated coagulation in a factor XII-dependent manner. Polyphosphate content correlated with the procoagulant activity of prostasomes. Inherited deficiency in factors XI, XII or high molecular weight kininogen, but not plasma kallikrein, protected mice from prostasome-induced lethal pulmonary embolism. Targeting polyphosphate or factor XII conferred resistance to prostate cancer-driven thrombosis in mice, without increasing bleeding. Inhibition of factor XII with recombinant 3F7 antibody reduced the increased prostasome-mediated procoagulant activity in patient plasma.

The data illustrate a critical role for polyphosphate/factor XII-triggered coagulation in prostate cancer-associated thrombosis with implications for anticoagulation without therapy-associated bleeding in malignancies.
INTRODUCTION

Cancer is an independent and major risk factor for venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE). Of all first VTE events 20-30% are malignancy-associated and VTE is the second leading cause of death in patients with malignancy. Anticoagulation therapy in cancer patients remains challenging with high recurrence rates of VTE and increased rates of anticoagulant-related bleeding. Currently used anticoagulants such as low molecular weight heparin (LMWH) and vitamin K antagonists (VKAs), target enzymes of the coagulation cascade that are critical for fibrin formation. As a result, treatment of VTE carries an inherent risk of potentially life-threatening bleeding. Prostate cancer (PC) is the second most common cancer in men and ranks 6th in malignancy-related mortality. Although the incidence of approximately 13 malignancy-associated VTE cases per 1000 person-year is not particularly high in PC patients due to the high prevalence of the disease, concurrence of VTE and PC presents a major medical burden.

Fibrin formation is initiated in plasma by two distinct mechanisms, termed the extrinsic and intrinsic coagulation pathways. The extrinsic coagulation pathway is initiated by binding of circulating coagulation factor VII/VIIa to the transmembrane protein tissue factor (TF). In contrast, the intrinsic pathway of coagulation is triggered by contact-induced autoactivation of zymogen factor XII (FXII), resulting in the active protease FXIIa. FXIIa leads to fibrin formation via its substrate factor XI (FXI). Ablation of F12 and F11 genes protects mice from DVT and PE and inherited deficiency in FXI reduces the incidence of DVT in patients. Although targeting FXII interferes with thrombus formation in non-human primates, there is a lack in epidemiological studies that analyzed protection from thromboembolic disease in individuals with severe FXII deficiency. Procoagulant platelet-released polyphosphate (polyP) activates FXII in vitro with implications for thrombosis in vivo. PolyP is a linear, unbranched polymer of orthophosphate residues linked by phosphoanhydride bonds. The polymer is ubiquitously found in nature and varies in chain length from a few phosphate units to several thousands.

The principle fibrin forming mechanism underlying cancer-associated thrombosis is considered to be up-regulation of TF expression on cancer cells and cancer cell-derived membrane vesicles. Indeed, clinical and experimental studies revealed largely increased TF antigen on PC cells and secreted exosomes (prostasomes), in tumor tissue and in plasma samples of PC patients, which was associated with excess activity of the extrinsic coagulation pathway. Prostasomes released from large intracellular storage vesicles of prostate epithelial cells were originally described in seminal fluid and are procoagulant in plasma. Prostasomes share cholesterol- and sphingomyelin-rich plasma membranes with other exosomes secreted by pancreatic, breast or colon adenocarcinoma cells.

Here, we identify a novel and unexpected role of the polyP/FXII-driven intrinsic pathway of coagulation in PC-associated thrombosis. Coagulation analyses of patient plasma and PE models in genetically altered mice show that PC cells and prostasomes expose long chain polyP on their surface. The polymer activates FXII, triggers clotting in PC patient plasma and causes thrombosis in mice. Interference with the polyP/FXII pathway provides protection from thrombosis while not increasing bleeding risk. These data identify a new coagulation mechanism that contributes to PC-driven thrombosis and suggest that interference with the polyP/FXII axis constitutes a novel target for anticoagulant drug development in PC-related thrombosis without impairing hemostasis.
METHODS

Prostasome-induced pulmonary thromboembolism
Mice were anesthetized by intraperitoneal (i.p.) injection of 2,2,2-tribromoethanol and 2-methyl-2-butanol. PC3 cell- [ATCC: CRL-1435; 0.8 µg/g body weight (bw)], seminal- (10 µg/g bw) or patient-derived prostasomes (150 µg/g bw) were mixed with epinephrine (0.06 µg/g bw) and slowly injected into the inferior vena cava. In some experiments, mice were injected intravenously (i.v.) with active site inhibited factor VII (ASIS; 2.5 µg/g bw), 3F7 (4.5 µg/g bw), polyP-binding domain (PPBD) of Escherichia coli (E. coli) exophosphatase (EC number 3.6.1.11; 150 µg/g bw) or saline 10 min before challenge. None of these inhibitors increased bleeding. Lung perfusion, bleeding time and loss of hemoglobin assays were previously described. Systemic blood pressure was measured by volume pressure recording of the tail, using the CODA non-invasive blood pressure system (Kent Scientific Corp., Torrington, CT, USA).

Supplemental methods
Detailed methods describing study design, blood collection, animals, cell culture, prostasome/exosome isolation, extraction of polyP, electron microscopy, expression of PPBD, ELISA for polyP detection, staining of polyP, immunofluorescence, real-time thrombin generation analysis, recalcification time and activation of FXII, histological analysis, and data analysis are available in the Supplemental Methods section on the Blood website.
RESULTS

Prostasomes initiate pulmonary embolism via the intrinsic pathway of coagulation

To analyze the functions of prostasomes in venous thrombosis, we challenged mice in a model of lethal PE by infusion of human PC cell (PC3)-derived prostasomes into the inferior vena cava. Mice that survived the challenge >30 minutes were considered survivors. A single animal out of 15 wild-type (WT) mice survived prostasome injection (Fig. 1A). In contrast, FXII deficient (F12−/−) mice were significantly protected from prostasome-induced PE and 5/6 survived (P < .001 F12−/− vs. WT). Mice deficient in FXI (F11−/−) were also protected (6/6 survived, P < .001 F11−/− vs. WT, P > .05 F11−/− vs. F12−/−). In addition to FXI, FXII cleaves plasma prekallikrein (PPK) to generate plasma kallikrein (PK) that liberates the inflammatory mediator bradykinin (BK) from its precursor high molecular weight kininogen (HK). We analyzed HK (Kng1−/−) and PPK (Klkb1−/−) deficient mice in our PE model. Kng1−/− mice were protected from prostasome-induced challenge (5/5 survived, P < .01 Kng1−/− vs. WT), and the survival rate was similar to that observed in F12−/− and F11−/− mice (P > .05 Kng1−/− vs. F12−/−, P > .05 Kng1−/− vs. F11−/−). In contrast, Klkb1−/− mice were as susceptible to prostasome-driven PE as WT animals (1/5 survived, P > .05 Klkb1−/− vs. WT). Kinin B2 receptor deficient (Bdkrb2−/−) mice are resistant to BK signaling. 5/6 Bdkrb2−/− mice died upon prostasome injection (P > .05 Bdkrb2−/− vs. WT), indicating that low BK is not contributing to the protection from thrombosis observed in F12−/− mice. Consistent with increased TF-dependent procoagulant activity in plasma of PC patients,25 targeting TF activity with ASIS (2.5 µg/g bw) protected WT mice from prostasome-induced PE (5/6 survived, P < .01 WT+ASIS vs. WT). We determined lung perfusion from prostasome-treated mice by i.v. administration of Evans blue dye (Fig. 1B). Perfused lung areas turned blue while occluded parts remained a natural reddish color. Consistent with prostasome-induced lethal PE, prostasome challenge in WT mice resulted in lung vessel occlusion visualized by disturbed perfusion of Evans blue. In contrast, lungs of F12−/−, F11−/− or WT mice pretreated with ASIS presented with uniform distribution of Evans blue. Histological sections of lungs from prostasome-challenged mice probed with the fibrin-specific antibody 59D8 (that does not cross-react with fibrinogen), and quantification of formed thrombi supported a role for FXII in prostasome-driven venous thrombosis (Figs. 1C and 1D). While fibrin deposition was seen throughout the pulmonary vasculature of WT mice (dead and survivors), virtually no thrombi were found in F12−/−, F11−/− and WT mice treated with ASIS. Fibrin detection in lung tissue by immunoblot analysis confirmed reduced fibrin accumulation in lungs of F12−/− mice, F11−/− mice and ASIS-treated WT mice as compared to untreated WT animals (Fig. 1E). As cell culture-derived prostasomes might differ from those generated in vivo, we isolated prostasomes from human seminal fluid19 and analyzed them in our PE model. Infusion of seminal prostasomes induced lethal PE in 5/6 WT mice (Fig. 1F). In contrast, F12−/− (4/6 survived), F11−/− (4/6 survived) and ASIS-treated WT (3/6 survived) mice were largely protected from seminal prostasome-triggered thrombotic challenge (P < 0.05 vs. WT, each). Consistently, prostasomes isolated from PC patient plasma also induced lethal PE in WT mice (0/5 survived, P < .01; Fig. 1G). In contrast, all WT mice (5/5) survived challenge with exosomes isolated from healthy men.

Prostasomes trigger FXII-mediated thrombin and fibrin formation in vitro

We characterized human seminal- and PC3 cell culture-derived prostasomes using transmission electron microscopy (TEM). Both seminal- (Fig. 2A) and cell culture- (Fig. 2D) prostasomes appeared as cup-shaped vesicles due to dehydration. The particles shared a similar size distribution with minimal and maximal diameter of 30 and 400 nm [Figs. 2B (seminal) and 2E (cell culture-derived)]. Consistent with earlier findings,19 the majority of prostasomes were within a size range of 30-200 nm. Less than 3% (seminal) and 6% (cell) of the analyzed prostasomes had a diameter >200 nm. Real-time thrombin generation in plasma using the calibrated automated thrombography (CAT) method analyzed the procoagulant activity of seminal and cell culture prostasomes. Seminal (Fig. 2C) and cell culture (Fig. 2F) prostasomes induced thrombin generation in a dose-dependent manner. Prostasomes shortened lag time and time to peak and increased maximal and total thrombin (endogenous thrombin potential, ETP). Prostasome-triggered thrombin formation was similar
in normal platelet-free and vesicle-depleted plasma. Deficiency in factors XII and XI and addition of the recombinant FXIIa-inhibitor rHA-Infestin-4 (Inf-4, 500 µg/ml) to normal plasma significantly prolonged lag time and time to peak, and reduced peak and total thrombin activated with prostasomes (Fig. 2G, Table 1). Inhibition of TF activity with ASIS addition. Prostasome-induced thrombin formation was more reduced in FXI- than in FXII-deficient plasma (ETP: 568 ± 176 vs. 1098 ± 186 nM*min). In the absence of FXI, TF-mediated thrombin generation is unable to amplify coagulation via the FXI feedback activation loop. Combined application of Inf-4 and ASIS completely blunted prostasome-induced procoagulant activity (Fig. 2H, Table 1). Consistent with thrombin formation assays, prostasomes initiated plasma clotting in a FXII-dependent manner (Fig. 2I). Addition of Inf-4 prolonged prostasome-stimulated recalcification clotting time vs. buffer in ultracentrifuged normal plasma (NP) (766 ± 55 sec vs. 357 ± 35 sec, P < .001). In line, prostasome-initiated clotting was prolonged in plasma deficient in factors XII (594 ± 53 sec, P < .01 vs. NP) or XI (943 ± 31 sec, P < .001 vs. NP). Addition of ASIS to NP also interfered with prostasome-induced clotting (546 ± 46 sec, P < .01 vs. NP). Combined application of Inf-4 and ASIS completely abolished prostasome-initiated fibrin formation (>1500 sec). The FXIIa/PK chromogenic substrate S-2302 confirmed that prostasomes dose-dependently initiated the contact system in NP (Fig. 2J). Prostasome-induced S-2302 conversion was reduced to buffer levels in FXII deficient plasma (P > .05) and was largely diminished in the absence of PPK or HK (P < .05 vs. NP+Buffer, Fig. 2K). Similarly to PC3 prostasomes, exosomes derived from pancreatic cancer (Panc1, BxPC3 and Capan2) and promyelocytic leukemia cells (HL-60) triggered S-2302 cleavage in a FXII dependent manner (Fig. 2L).

**Prostasome- and PC cell-derived polyP initiates fibrin formation via FXII activation**

How do prostasomes and PC cells generate FXIIa? One possible candidate is the FXII contact activator polyP. We purified polyP from PC3 prostasomes (Fig. 3A) and PC3 cells (Fig. 3B) by anion affinity chromatography. Eluted material was separated by urea/acylamide-gel electrophoresis and visualized by the polyP-sensitive DAPI-negative staining. Prostasome and PC3 cell polyP migrated with similar heterodispersity and polymer chain length ranging from approximately 200 to >1000-mers. Treatment with phosphatase (Psp), which hydrolyzes polyP, completely abolished the signal. We used recombinant PPBD as a probe for polyP. PPBD specifically binds to polyP of chain length >35. PolyP localized to the surface of prostasomes and co-localized with the prostasome cell-surface marker CD63 by anion affinity chromatography. Eluted material was separated by urea/acylamide-gel electrophoresis and visualized by the polyP-sensitive DAPI-negative staining. Prostasome and PC3 cell polyP migrated with similar heterodispersity and polymer chain length ranging from approximately 200 to >1000-mers. Treatment with phosphatase (Psp), which hydrolyzes polyP, completely abolished the signal. We used recombinant PPBD as a probe for polyP. PPBD specifically binds to polyP of chain length >35. PolyP localized to the surface of prostasomes and co-localized with the prostasome cell-surface marker CD63 (Fig. 3C). Treatment of prostasomes with Psp blunted PPBD binding (not shown). Similarly to prostasomes, polyP enriched at the plasma membranes of non-permeabilized PC3 cells (Fig. 3D). PPBD detected cytoplasmic polyP in a spotty vesicular pattern in permeabilized PC3 cells (Fig. 3F). We incubated prostasomes with human plasma and stained for FXIIa using Alexa 488-conjugated 3F7 antibody that specifically cross-reacts with the active enzyme but not with the zymogen form. Plasma incubation led to FXIIa formation and the protease co-localized with polyP at the plasma membrane (Fig. 3E). PolyP isolated from prostasomes (Fig. 3G) and PC3 cells (Fig. 3H) stimulated S-2302 conversion in plasma. The polyP inhibitors PPBD (1 mg/ml), generation 1.0 cationic poly(amide amine) (PAMAM) dendrimer (25 µg/ml) and degradation of polyP with Psp (1 U/ml) abolished polyP-induced amidolytic activity. PolyP was inactive in FXII deficient plasma. Consistent with PC polyP-stimulated FXII formation, prostasome- (Fig. 3I) and PC3 cell- (Fig. 3J) polyP triggered thrombin formation in a FXII dependent manner and PPBD and generation 1.0 cationic PAMAM dendrimer blocked the procoagulant activity conferred by PC-derived polyP. To analyze the functions of PC-derived polyP in thrombosis, we injected WT mice i.v. with saline or PPBD (150 µg/g bw) 10 min prior to prostasome challenge (Fig. 3K). PPBD significantly protected mice from lethal PE, whereas all saline-infused animals died within 30 min after challenge (3/5 vs. 0/5 mice survived, P < .05). We analyzed polyP on prostasome plasma membranes using a PPBD-based ELISA (Fig. 3L). PolyP content decreased from PC3- over seminal- to patient plasma-derived prostasomes. PolyP content correlated with the procoagulant activity of prostasomes. We analyzed histological sections of human prostate adenocarcinoma for the procoagulant polyP/FXII pathway (Figs. 3M-P’).
Immunohistochemistry showed FXII antigen in the lumen of occluded blood vessels. Consistent with ectopic FXII expression by ovarian cancer cells, the blood coagulation factor was detected in PC tissue (Figs. 3N and 3N'). We probed for polyP and fibrin in PC tissue frozen sections as the water-soluble polyP gets lost during the deparaffinization procedure. PPBD showed polyP enriched in cancer glands and in stromal smooth muscle fibres (Figs. 3O and 3O') and 59D8 antibody visualized extravascular fibrin deposition at these sites (Figs. 3P and 3P').

**FXIIa inhibition provides safe protection from prostasome-induced lethal pulmonary embolism**

The fully humanized anti-FXIIa antibody 3F7 is a candidate future therapeutic agent, binds into the FXIIa enzymatic pocket and inhibits the amidolytic activity of the protease. 24 3F7 dose-dependently interfered with synthetic long chain polyP (LC polyP)-stimulated thrombin generation and antibody concentrations ≥375 nM completely abolished polyP-triggered coagulation in murine plasma (Fig. 4A). To analyze the prophylactic application of 3F7 for interference with PC-mediated PE, we injected WT mice i.v. with saline or 3F7 (4.5 µg/g bw) 10 min prior to prostasome challenge (Fig. 4B). 3F7 conferred significant protection (P< .001 vs. saline) from prostasome-induced PE (6/6 mice survived) whereas all saline-infused animals, with the exception of a single mouse, died within 30 min after challenge. Increased survival rate in 3F7-treated mice correlated with increased lung perfusion and less vascular occlusion compared to lungs of saline-treated animals (Fig. 4C). Fibrin staining in histologic sections of lungs from saline-treated mice confirmed diffuse thrombotic occlusion, while fibrin deposition was significantly less (P< .001 vs. saline) in 3F7-infused animals (Fig. 4D and 4E). We determined the tail bleeding time and extent of bleeding (blood loss) by quantification of lost hemoglobin until cessation of bleeding as a measure of hemostatic capacity. 3F7 neither prolonged the bleeding time (270 ± 117 sec vs. 282 ± 148 sec, P>.05, Fig. 4F) nor increased hemoglobin loss (A575 of 0.63 ± 0.22 vs. 0.68 ± 0.38, P>.05, Fig. 4G) compared to saline-treated mice.

**Activation of FXII contributes to increased procoagulant activity of PC-derived plasma prostasomes**

Thrombin generation was increased in plasma samples from PC patients (Suppl. Table 1) over levels in healthy men confirming previous studies. Lag time (26.7 ± 5.1 vs. 43.7 ± 4.7 min, P< .05, Fig. 5A) and time to peak (30.2 ± 4.7 vs. 47.6 ± 3.7 min, P< .05, Fig. 5B) were shortened, and maximum (122 ± 27.4 vs. 29.9 ± 10.7 nM, P< .05, Fig. 5C) and total thrombin (859.7 ± 183.9 vs. 355.2 ± 136.8 nM*min, P< .05, Fig. 5D) were significantly increased in patients over control. Depletion of vesicles abolished the procoagulant activity in patient plasma and reconstitution of the ultracentrifuged plasma samples with purified prostasomes restored their procoagulant activity. We analyzed the contribution of prostasome polyP-generated FXIIa for procoagulant activity in PC patients. Prostasomes/exosomes isolated from plasma of 20 PC patients, 10 healthy male and 10 healthy female controls (Suppl. Table 2) triggered thrombin formation in normal plasma. Exosomes derived from female plasma exhibited procoagulant activity similar to that of healthy males (ETP: 938 ± 114 vs. 683 ± 121 nM*min, P>.05). For comparison we supplemented ultracentrifuged plasma with synthetic phospholipids. Procoagulant activity conferred by phospholipids alone was similar to those induced by healthy individuals (ETP: 1073 ± 106 nM*min, P>.05 vs. healthy males and females). The procoagulant activity of prostasomes from PC patients was significantly higher than those from healthy male controls. Lag time (25.1 ± 2.1 vs. 14.5 ± 1.1 min, P< .001, Fig. 5E) and time to peak (32.0 ± 1.9 vs. 20.8 ± 1.3 min, P< .001, Fig. 5F) were shortened by 42% and 35%, respectively, and maximum (39 ± 7 vs. 145 ± 14 nM, P< .001, Fig. 5G) and total thrombin (683 ± 121 vs. 1763 ± 39 nM*min, P< .001, Fig. 5H) were increased by 3.7- and 2.6-fold, respectively. Inhibition of FXIIa activity with 3F7 (100 µg/ml) significantly reduced the increased procoagulant activity of patient prostasomes. 3F7 prolonged lag time (22.0 ± 2.2 min, P< .01 vs. w/o 3F7) and time to peak (28.2 ± 2.2 min, P< .01 vs. w/o 3F7). Consistently, 3F7 reduced peak (95 ± 16 nM, P< .05 vs. w/o 3F7) and total thrombin (1270 ± 125 nM*min, P< .001 vs. w/o 3F7) in normal plasma samples.
supplemented with prostasomes from PC patients. FXIIa inhibition similarly reduced procoagulant activity of patient- and cell culture-derived prostasomes (Fig. 2G, Table 1, 38% ETP reduction each). Procoagulant activity of prostasomes isolated from PC patients with a history of thromboembolic disease was increased over samples from patients without previous vascular occlusive events (Suppl. Fig. 1). Taken together, these data show a role of prostasome-generated FXIIa in the increased procoagulant activity seen in PC patients.
DISCUSSION

Malignancy has been associated with increased risk of thrombosis for more than a century. The TF/factor VIIa pathway is believed to be the principal initiator of fibrin formation in cancer patients. Cancer cells express TF on their plasma membrane and release TF-bearing microparticles (MP) into the circulation. MPs are procoagulant as they promote thrombus propagation in mouse models and trigger clotting in human plasma. In various types of cancer, including PC, plasma MP number and procoagulant activity are increased supporting a role of TF-bearing MP in malignancy-associated thrombosis. Indeed, some studies show that MP-associated TF activity is increased in cancer patients with VTE over asymptomatic cancer patients and impedance-based flow cytometry established TF-bearing MP as a prognostic risk factor for VTE. However, other studies that analyzed plasma MP counts indirectly via prothrombinase assays or MP TF activity could not establish an association between MP levels/TF activity and risk for VTE. Furthermore, plasma TF activity in cancer patients does not correlate with the MP count and plasma TF activity in cancer patients does not correlate with other biomarkers of thrombosis, including D-dimer or thrombin-antithrombin complexes. Collectively, clinical data suggest that mechanisms other than the TF pathway operate and contribute to thrombosis in cancer patients.

Here, we show that particles derived from PC, pancreatic cancer as well as promyelocytic leukemia cells initiate coagulation in a FXII-dependent manner, triggered by exposure of polyP on their plasma membranes. These findings extend earlier studies showing that MPs derived from platelets of healthy individuals initiate coagulation via FXII activation. Vice versa platelet-stimulated fibrin formation is defective in the absence of FXII. Supporting a function for the polyP/FXII coagulation pathway in cancer-associated thrombosis, zymogen FXII plasma levels are low in samples from patients with advanced gastrointestinal and non-metastatic colorectal cancer compared to healthy controls. Furthermore, diminished plasma FXII is associated with reduced PPK levels and an increase in PK bound to its endogenous protease inhibitors in lung cancer patient plasma samples. These observations suggest that polyP/FXII-initiated coagulation has implications in patients with PC and also in patients with other malignancies.

PolyP procoagulant activity increases with chain length and polymers of <45 units do not support FXII contact activation. Consistent with synthetic long chain polyP, natural PC-derived polyP (200 to >1000-mers) is a potent FXII contact activator. PolyP content exposed on the plasma membrane of various prostasomes varied and correlated with the procoagulant activity of these particles. There was a trend for higher polyP expression on prostasomes derived from PC patients with a history of thromboembolic disease, however, further studies are warranted to confirm this observation. In addition to initiating the FXIIa-driven intrinsic pathway of coagulation, polyP accelerates multiple downstream procoagulant mechanisms including factor V and factor XI feedback activation, and blocks the anticoagulant activity of TF pathway inhibitor (TFPI). The polyP/FXII pathway operates independently of TF-mediated coagulation and also results in PK mediated release of the proinflammatory and vasodilatory peptide BK. Therefore, targeting polyP using cationic proteins, polymers, or small molecules may represent a promising strategy for interfering with cancer-driven coagulation and potentially, inflammatory reactions. PolyP/FXII inhibition and deficiency in factors XI, XII or HK interfered with prostasome-induced lethal PE. Similar to infusion of pure polyP and platelet stimulation, injection of prostasomes induced microvascular PE. In humans, microvascular PE represents a complication of tumor cell embolism. Klkb1−/− mice died upon i.v. pure polyP infusion. The animals were not protected from prostasome challenge either, supporting the presence of polyP on prostasomes. Infusion of polyP into Bdkrb2−/− animals induced lethal PE and consistently, the mice were also susceptible to prostasome-triggered PE. Although polyP initiates BK formation, i.v. prostasome infusion did not reduce systemic blood pressure. A plausible explanation is that formed BK is rapidly inactivated in the pulmonary microvasculature by...
kinin-degrading enzymes before the mediator reaches pressure-regulating vessels.\textsuperscript{61-64}

Our animal models and patient material data show that both TF/FVIIa and polyP/FXIIa pathways contribute to PC-associated hypercoagulable states. Thrombin formation \textit{in vitro} was more reduced in TF- than in FXIIa-inhibited plasma (\textbf{Fig. 2}), however, targeting each of the pathways was sufficient to protect mice from lethal PE \textit{in vivo} (\textbf{Fig. 1}). TF/FVIIa and polyP/FXIIa have distinct and differential functions in thrombus initiation and propagation. TF appears as the initiator of thrombosis on ruptured plaques, while FXIIa drives coagulation distant from the vessel wall within the propagating thrombus.\textsuperscript{65} Indeed, inhibition of FXII activity interferes with mechanical thrombus stability in whole blood\textsuperscript{15} and increases embolization rate in treated mice.\textsuperscript{65} Emphasizing a role for FXII in thrombus growth, inherited deficiency or pharmacologic neutralization of the factor is sufficient to protect mice from TF-triggered lethal PE.\textsuperscript{66}

In cancer-related thrombosis, LMWH is the preferred mode of anticoagulation over VKAs (warfarin).\textsuperscript{67} This recommendation is based on data showing decreased VTE recurrence with use of LMWH compared to VKAs.\textsuperscript{68} In addition, VKA dosing in cancer patients is challenging as clinical studies indicated that the incidence of bleeding remains high even when INR values being close to or below 2.0.\textsuperscript{69,70} Bleeding complications are not restricted to VKA therapy as prospective randomized controlled trials comparing LMWH and VKA therapy in cancer-associated VTE have reported similar bleeding rates.\textsuperscript{68} Data are insufficient to recommend the routine use of new oral anticoagulants as therapy for VTE in cancer patients.\textsuperscript{71} Due to the potentially serious and life-threatening bleeding, all patients require an individualized assessment of their bleeding risk prior to the initiation of anticoagulation. Thus, there is need for effective anticoagulation strategies for patients with cancer. Targeting polyP/FXII interfered with the increased procoagulant activity in patient plasma (\textbf{Fig. 5}) and prevented PC-driven PE in mice without treatment-related risk of bleeding (\textbf{Figs. 3 and 4}). Importantly, the decisive role of the intrinsic pathway of coagulation in thrombosis is not restricted to murine models. We have recently shown that the FXIIa-inhibitory antibody 3F7 offers a safe thromboprotective strategy in a preclinical setting.\textsuperscript{24} 3F7 blocks thrombosis as effectively as heparin in a cardiopulmonary bypass system in rabbits (ECMO system). However, in sharp contrast to heparin, 3F7 did not impair the hemostatic capacity of treated animals. Fully humanized 3F7 antibody has an immediate anticoagulant effect and is thus suitable for management of VTE in an acute setting.\textsuperscript{24} Humanized antibodies expressed in mammalian cells are an important class of human therapeutic products and are expected to display minimal immunogenic potential in humans.\textsuperscript{72,73} An alternative method to interfere with FXII function is the use of antisense oligonucleotides (ASO) that knockdown FXII expression and confers protection from thrombosis without an increase in therapy-associated bleeding.\textsuperscript{74,75} Furthermore, lowering FXI levels with FXI-ASO reduced the rate of postoperative thrombosis and was safe with respect to bleeding risk in patients.\textsuperscript{76} A disadvantage of this approach, however, is the long lag time in achieving therapeutic levels of anticoagulation (injections required 1-3 times weekly for 5 weeks). Currently, small molecule FXIIa inhibitors are under development with potential anticoagulant and additional anti-inflammatory activities.

In summary, the current study identifies a novel procoagulant mechanism driven by long chain polyP on PC cells and prostasomes that contributes to clotting in patient plasma \textit{in vitro} and PE in mouse models \textit{in vivo}. Interference with the polyP/FXII pathway provides a new approach for anticoagulation in PC-associated thrombosis that lacks the bleeding risk of currently used anticoagulants.
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AUTHORSHIP CONTRIBUTION

K.F.N. and L.L. performed most of the experiments; F.L., C.B., G.S., M.G., provided clinical material and analyzed patient samples. G.R. and G.R. provided tools and materials to isolate and characterize prostasomes; N.M. and T.A.F. discussed content and contributed to the experimental design; T.R. provided grant support, designed the experiments and wrote the manuscript with E.X.S. and K.F.N. All authors discussed the results and commented on the manuscript.

DISCLOSURE OF CONFLICT OF INTEREST

The authors have no conflicting financial interests.
REFERENCES


**TABLE 1. Prostasome-driven thrombin generation.** Comparison of PC3 prostasome-induced thrombin formation in platelet free normal plasma (NP), NP supplemented with rHA-Infestin-4 (Inf-4; 500 µg/ml), ASIS (30 nM) or a combination of ASIS and Inf-4 (30 nM and 500 µg/ml) and plasma deficient in FXII (FXII def.) or FXI (FXI def.) as done in Figs. 2G and 2H. Values are expressed as mean ± SEM, n=3-11. *P< .05, **P< .01 and ***P< .001 vs. buffer-treated NP, ANOVA. ETP, endogenous thrombin potential.

<table>
<thead>
<tr>
<th></th>
<th>NP</th>
<th>NP+Inf-4</th>
<th>FXII def.</th>
<th>FXI def.</th>
<th>NP+ASIS</th>
<th>NP+Inf-4+ASIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>10.7 ± 1.0</td>
<td>22.0 ± 3.7***</td>
<td>16.0 ± 2.5</td>
<td>21.0 ± 2.0</td>
<td>20.6 ± 1.9***</td>
<td>n.a.</td>
</tr>
<tr>
<td>Time to peak (min)</td>
<td>15.2 ± 1.1</td>
<td>30.1 ± 5.0***</td>
<td>25.3 ± 2.7***</td>
<td>27.9 ± 2.1***</td>
<td>33.3 ± 4.1***</td>
<td>n.a.</td>
</tr>
<tr>
<td>Peak thrombin (nM)</td>
<td>191.1 ± 16.0</td>
<td>109.4 ± 31.3***</td>
<td>94.3 ± 20.1***</td>
<td>41.6 ± 14.5***</td>
<td>51.9 ± 12.8***</td>
<td>n.a.</td>
</tr>
<tr>
<td>ETP (nM*min)</td>
<td>1622 ± 59</td>
<td>998 ± 267</td>
<td>1098 ± 186*</td>
<td>568 ± 176***</td>
<td>686 ± 157***</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

n.a. below the detection limit
FIGURE LEGENDS

FIGURE 1. Prostasomes initiate lethal PE in mice via the intrinsic coagulation pathway. (A) PE was induced by i.v. infusion of PC3 cell-derived prostasomes [0.8 μg/g body weight (bw)] in WT, F12<sup>−/−</sup>, F11<sup>−/−</sup>, Kng1<sup>−/−</sup>, Klkb1 <sup>−/−</sup>, and Bdkrb2<sup>−/−</sup> mice or WT animals pretreated with ASIS (WT+ASIS; 2.5 μg/g bw). Mortality was assessed in each group of mice (n=5-15). Animals alive 30 min after challenge were considered survivors. Horizontal bars represent mean-values. ***P < .001 and **P < .01 vs. untreated WT. (B) Prostasome challenged mice were i.v. infused with Evans blue shortly after the onset of respiratory arrest while the heart was still beating, or after 30 minutes for those animals that survived. Lungs were excised and perfusion defects were analyzed. (C) Immunohistochemical localization of fibrin deposition on sections from lungs of WT, F12<sup>−/−</sup>, F11<sup>−/−</sup> and WT+ASIS-treated mice using the fibrin-specific antibody 59D8 (high magnification, lower right). Sections were counterstained with Mayer’s hematoxylin (bar=100 µm). (D) Thrombi per visual field were counted at 10x magnification from sections such as those in (C). Columns are mean ± SEM for 35 fields. (E) Accumulation of fibrin in lungs of prostasome-challenged WT, F12<sup>−/−</sup>, F11<sup>−/−</sup> and WT+ASIS mice was analyzed by immunoblotting. (F, G) Pulmonary thromboembolism model as in (A) induced by injection of 10 μg/g bw seminal prostasomes (F, n=6) or 150 μg/g bw healthy male or patient prostasomes/exosomes (G, n=5), *P < .05 vs. untreated WT (F) and **P < .01 vs. healthy males (G). P values were determined using one-way analysis of variance (ANOVA); n.s., nonsignificant.

FIGURE 2. Prostasomes trigger coagulation in a FXII-dependent manner. Comparison of seminal (A-C, upper panel) and PC3 cell prostasomes (D-F, lower panel): Transmission electron microscopy (TEM) images show morphologies of isolated seminal (A) and PC3 (D) prostasomes (bar=500 nm). Size distributions of seminal (B) and PC3 (E) prostasomes assessed from TEM images as shown in (A) and (D), 6 fields each. Real-time thrombin generation stimulated with increasing concentrations (0-28 µg/ml) of seminal (C) and PC3 (F) prostasomes in platelet free (solid lanes ) and ultracentrifuged (dashed line) plasma. Representative curves of a series of n=6 are shown. (G, H) Mechanism of prostasome-driven coagulation: Real-time thrombin generation initiated by 14 µg/ml PC3 prostasomes in normal plasma (NP), NP supplemented with rHA-Infestin-4 (Inf-4; 500 µg/ml), ASIS (30 nM) or a combination of ASIS and Inf-4 (30 nM and 500 µg/ml) and plasma deficient in FXII (FXII def.) or FXI (FXI def.), n=3-11. (I) PC3 prostasomes (1.75 µg/ml)-stimulated recalcification clotting time in ultracentrifuged NP in the presence of inhibitors as in (G, H) or buffer, or in FXII- or FXI-deficient plasma. **P < .01 and ***P < .001 vs. NP, n=5. (J-L) Contact activation was analyzed by conversion of the FXIIa/PA chromogenic substrate S-2302. Buffer stimulated plasma is shown as control. (J) Plasma was incubated with increasing concentrations of PC3 prostasomes (0-200 µg/ml). (K) PC3 prostasomes (100 µg/ml)-induced S-2302 conversion in NP or plasma deficient in FXII (FXII def.), prekallikrein (PPK def.) or high molecular weight kininogen (HK def.). *P < .05 and **P < .001 vs. NP+Buffer. Bars represent the absorbance at λ=405 nm at 60 min, n=6. (L) Normal (dark columns) or FXII deficient (light columns) plasma was incubated with PC3 prostasomes and exosomes from various cancer cells (Panc1, BxPC3, Capan2 and HL-60, 100 μg/ml each). Absorbance of cleaved S-2302 in prostasome treated plasma is given relative to buffer signal at 60 min. ***P < .001 NP vs. FXII def., n=3-6. P values were determined using one-way analysis of variance [ANOVA (I, K)] or Student’s t test (L). Data are presented as mean ± SEM; n.s., nonsignificant.

FIGURE 3. Prostasomes, PC cells and PC tissue expose procoagulant polyP. Isolation of polyP from prostasomes and PC cells: (A, B) PolyP was extracted from PC3 prostasomes (A) and cells (B) by an anion exchanger chromatography, separated by electrophoresis on polyacrylamide/urea gel and visualized by DAPI negative staining. Synthetic polyP with mean chain length of 134, 383 and 637 serve as molecular size standard. Purified polyP and synthetic long chain (LC) polyP were loaded prior and after incubation with phosphatase (Psp, 10 U/ml for 1 h). (C, E) Epifluorescence images of polyP on PC3 prostasomes using...
Alexa 594-labeled PPBD. The polymer co-localizes with prostatic surface membrane marker CD63 (anti-CD63, C) and FXIIa (3F7, E). (D, F) Confocal laser scanning microscopy of polyP in non-permeabilized (D) and permeabilized (F) PC3 cells. PolyP is stained with Alexa 488-conjugated PPBD and DNA was visualized with DAPI. Bar is 500 nm in C and E and 10 µm in D and F. (G, H) PC polyP activates FXII. Platelet free plasma was incubated with 1.5 µg/ml PC3 prostasome (G) or cell (H) polyP in the absence or presence of PPBD (1 mg/ml), generation 1.0 dendrimer (25 µg/ml) or Psp (1 U/ml). PolyP-treated FXII deficient plasma (FXII def.) and buffer-stimulated normal plasma (buffer) is shown as control. Hydrolysis of the chromogenic substrate S-2302 measures formed FXIIa and PK. Mean ± SEM, n=6. (I, J) Real-time thrombin formation generated in platelet free plasma stimulated with 1.5 µg/ml prostasome (I) or PC3 cell (J) polyP in the absence or presence of PPBD (0.5 and 2 mg/ml) or generation 1.0 dendrimer (25 µg/ml). Thrombin formation in polyP-activated FXII deficient plasma (FXII def.) and buffer-stimulated normal plasma is blotted for comparison. A representative thrombin generation curve of a series of n=6 is shown. (K) Mortality associated with i.v. injection of PC3 prostasomes (0.8 µg/g bw) in WT mice pretreated with saline or PPBD (150 µg/g bw) was assessed in each group (n=5); animals alive at 30 min after challenge were considered survivors. *P < .05 vs. saline, unpaired Student’s t test. (L) PolyP detection on seminal, PC3 cell and patient prostasomes using PPBD binding in an ELISA. Bars represent polyP content relative to seminal prostasomes, mean ± SEM, n=3. ***P < .001 vs. seminal prostasomes, one-way analysis of variance (ANOVA). Immunohistochemistry of FXII, polyP and fibrin in human PC tissue sections (M-P'). Hematoxylin/eosin-staining of the adenocarcinoma [arrows denote occluded vessels (M, M')]. Immunohistochemical localization of FXII using anti-FXII antibodies [arrows indicate FXII positive occluded vessels and cancer tissue, respectively (N, N')], polyP using recombinant PPBD as a probe [arrows (O, O')] and fibrin deposits using 59D8 antibody [arrows (P, P')] in paraffin (M-N') and cryo (O-P') sections. Black and yellow asterisk denotes tissue areas containing cancer cells and non-malignant tissue, respectively. Sections were counterstained with Mayer’s hematoxylin. Bars are 100 µm.

**FIGURE 4. FXIIa inhibition protects mice from prostasome-induced PE.** (A) Real-time thrombin formation in murine platelet free plasma stimulated with PC size synthetic polyP (LC polyP, 100 µg/ml) in the absence or presence of increasing concentrations of anti-FXIIa antibody 3F7 (n=3). Molar antibody concentrations are relative to plasma FXII (375 nM). (B) Mortality associated with i.v. injection of PC3 cell prostasomes (0.8 µg/g bw) in WT mice pretreated with saline or 3F7 (150 µg/g bw) was assessed in each group (n=6); animals alive at 30 min after challenge were considered survivors. ***P < .001 vs. saline, unpaired Student’s t test. (C) Mice challenged with prostasomes were i.v. infused with Evans blue shortly after respiratory arrest while lungs were still perfused. Excised lungs show perfusion defects in red. (D) Sections from lungs of saline- and 3F7-treated WT mice were analyzed for fibrin by immunohistochemistry with 59D8 antibody and counterstained with Mayer’s hematoxylin, bar=100 µm. (E) Thrombi per visual field were counted at 10x magnification from sections such as those in (D). ***P < .001 vs. saline, unpaired Student’s t test. Columns are mean ± SEM for 35 fields. (F, G) 3F7 treatment does not impair hemostatic capacity. (F) Tail bleeding times and (G) total hemoglobin loss assessed by hemoglobin absorbance at λ=575 nm was determined in saline- and 3F7-infused mice. Mean ± SD, n=10.

**FIGURE 5. Prostasome/FXIIa-driven increased procoagulant activity in PC patient plasma.** (A-D) Real-time thrombin generation in plasma of healthy individuals (Controls) and patients (Patients). Thrombin formation in prostasome-depleted [Patients (-)] and prostasome-reconstituted [Patients (+)] patient plasma is blotted for control, n=9 each. (E-H) Real-time thrombin generation in normal plasma stimulated by addition of prostasomes/exosomes (250 µg/ml) from healthy female and male controls (n=10 each) or PC patients (n=20). Patient prostasome-triggered coagulation was measured in the absence or presence of 3F7 (100 µg/ml). Lag time till thrombin formation starts (A, E), time to peak thrombin (B, F), maximum thrombin (peak thrombin, C, G) and total thrombin (endogenous thrombin potential, ETP; D, H). Each symbol represents an individual. Horizontal bars
Factor XII in prostate cancer

indicate mean values. *P < .05, **P < .01 and ***P < .001, unpaired Student’s t test; n.s., nonsignificant.
Figure 1
Figure 5
The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis

Katrin Faye Nickel, Göran Ronquist, Florian Langer, Linda Labberton, Tobias A. Fuchs, Carsten Bokemeyer, Guido Sauter, Markus Graefen, Nigel Mackman, Evi X. Stavrou, Gunnar Ronquist and Thomas Renné