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Altered plasma fibrin clot properties in patients with digestive tract cancers: Links with the increased thrombin generation ☆

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ABSTRACT

Objectives: Evidence indicates that cancer patients have increased thromboembolic risk. Given a potential role of prothrombotic clot phenotype in thrombosis, we investigated plasma fibrin clot properties in patients with digestive tract cancer (DTC).
Methods: We studied 44 consecutive patients with DTC, including 26 subjects with colorectal cancer, versus 47 controls matched for demographics and cardiovascular risk factors. We evaluated ex vivo plasma fibrin clot permeability (K_s), turbidimetry (lag phase and maximum absorbance, ΔAbs), and efficiency of fibrinolysis using 3 different measures (CLT, $t_{50\%}$ and maximum rate of D-dimer release from clots, D-D_{rate}).
Results: Patients with DTC had lower K_s (−11.5%, $p=0.016$), shorter lag phase (−5%, $p=0.019$), longer CLT (+17%, $p<0.001$) and $t_{50\%}$ (+8%, $p=0.031$), and reduced D-D_{rate} (−12%, $p<0.001$) compared with controls. After adjustment for potential confounders, thrombin–antithrombin (TAT) complexes were the independent predictor of CLT and $t_{50\%}$ in the patients, while K_s was independently associated with tissue plasminogen activator but not with TAT. In high grade tumor patients ($n=26$) compared with the remainder ($n=18$), K_s was lower ($p=0.004$), and lag phase shorter ($p=0.03$), while CLT ($p=0.012$) and $t_{50\%}$ ($p=0.008$) were longer, suggesting more profound unfavorable alterations in fibrin clots properties.
Conclusions: This study is the first to show that patients with DTC tend to form less permeable fibrin clots relatively resistant to lysis. Prothrombotic clot phenotype might represent a novel prothrombotic mechanism in cancer patients.

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Introduction

Venous thromboembolism (VTE) is the second leading cause of death in cancer patients. Compared to the non-cancer population, patients with malignancies have 7-fold increased risk of VTE [1]. In gastrointestinal cancers the risk of VTE is 20-fold higher than in the general population [1]. Available data suggest that arterial thrombosis is a rather rare finding among cancer patients [2]. An increased incidence of ischemic heart disease and intermittent claudication associated with worse outcomes has been described in cancer patients [3].

Mechanisms underlying the increased risk of VTE in cancer patients are complex and multifactorial. A number of abnormalities in blood coagulation in association with cancer have been demonstrated, including elevated fibrinogen [4] and thrombin generation [5], enhanced expression of tissue factor (TF) and cancer procoagulant. TF, which is produced constitutively by cancer cells [6], is a major trigger of blood clotting through interaction with activated coagulation factor (F)VII [7]. CP, activates FX independently of FVII, leading to increased thrombin generation. Tumor cells are able to produce both urokinase-type (uPA) and tissue-type plasminogen activator (tPA), plasminogen activator receptor (u-PAR), plasminogen activator inhibitor-1 (PAI-1) and PAI-2. Growing evidence indicates that the disturbed balance between plasminogen activators and inhibitors, in addition to its hemostatic role, is important in tumor invasion, progression and metastasis [8]. Proinflammatory cytokines, produced and released by cancer cells, can affect anticoagulant pathways in the endothelium by increased TF synthesis and downregulation of thrombomodulin (TM) expression, resulting in impaired activation of the protein C anticoagulant pathway [9]. It has been shown that

Abbreviations: APTT, activated partial thromboplastin time; BMI, body mass index; CLT, clot lysis time; CRP, C-reactive protein; D-D_{max}, maximum D-dimer levels in the clot lysis assay; D-D_{rate}, maximum rate of increase in D-dimer levels in the clot lysis assay; DTC, digestive tract cancer; F, factor; G, grade; INR, international normalized ratio; K_s, permeation coefficient; PAI-1, plasminogen activator inhibitor-1; RBC, red blood count; $t_{50\%}$, time required for a 50% decrease in clot turbidity; TAT, thrombin-antithrombin complexes; TF, tissue factor; TM, thrombomodulin; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; u-PAR, plasminogen activator receptor; VTE, venous thromboembolism; WBC, white blood count.

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cancer cell extracts can enhance platelet aggregation, platelet turnover and reduce platelet survival time [10]. Cancer treatment, including chemotherapy and radiotherapy, can enhance a hypercoagulable state and promote VTE [11].

Formation of fibrin clots relatively resistant to lysis represents the final step in blood coagulation. The architecture of the fibrin network depends on physical, biochemical, and genetic factors and changes under various pathological conditions [12]. Fibrin clots composed of compact, highly-branched networks with thin fibers are less susceptible to lysis [13]. Such altered plasma fibrin clot properties, including reduced clot permeability and susceptibility to lysis, have been observed in patients with previous idiopathic VTE [14] and arterial thromboembolic events, including acute or previous myocardial infarction [15,16] and ischemic stroke [17,18]. Similar prothrombotic fibrin clot phenotype has been demonstrated in diseases with enhanced inflammation, for example in chronic obstructive pulmonary disease [19]. To our knowledge, there have been no published reports on fibrin clot properties in cancer patients. Given increased risk of thromboembolic events in cancer patients and a potential role of prothrombotic fibrin clot phenotype in thrombosis, we sought to investigate plasma fibrin clot properties in patients with DTC.

Materials and methods

Patients

Between February 2011 and January 2012, 44 consecutive patients with DTC and 47 control subjects matched for age, sex and cardiovascular risk were enrolled in the study. All the DTC patients were admitted to 3rd Chair and Department of General Surgery of Jagiellonian University Medical College for scheduled surgical treatment. Among them there were 16 subjects with rectal cancer, 10 with colon cancer, 8 with gastric cancer, 8 with pancreatic cancer and 2 with small bowel cancer. The half of rectal cancer patients underwent preoperative radiotherapy. There were only 3 patients with stage I (according to The American Joint Committee on Cancer staging scheme [20]) disease, 4 with stage II, 19 with stage III disease and 18 with stage IV. Based on the World Health Organization grading scheme, there were 3 patients with grade 1 tumors (G1, low grade), 15 patients with grade 2 (G2, intermediate grade) and 26 patients with grade 3 (G3, high grade). The inclusion criterion was histologically confirmed DTC. Exclusion criteria were: any active infection, renal dysfunction (creatinine >2 mg/dl), hypo- and hyperthyroidism, previous myocardial infarction, stroke or another acute vascular event, VTE, known bleeding diathesis, current anticoagulant therapy except for low molecular weight heparin administered for the last time 12 h or more prior to sample collection.

The study protocol was approved by Jagiellonian University Bioethics Committee. A written consent was obtained from each participant.

Laboratory investigations

Fasting blood was taken from the antecubital vein between 7 and 9 AM. Routine laboratory tests were used to determine blood cell count, glucose, creatinine, protein, albumin, international normalized ratio (INR), activated partial thromboplastin time (APTT), C-reactive protein (CRP) and fibrinogen. Immunoenzymatic assays were used to determine in citrated plasma tPA antigen (American Diagnostica, Greenwich, CT, USA), PAI-1 antigen (Hyphen BioMed, Neuville-Sur-Oise, France) and TAT (Siemens, Marburg, Germany).

Blood for fibrin clot analysis was collected into tubes containing 0.109 M trisodium citrate and centrifuged within 20 min at 1500 g. Plasma was frozen and stored at -80 °C until analysis. Technicians blinded to the origin of the samples performed all measurements. Intra-assay and inter-assay coefficients of variation were <8%.

Fibrin clot permeability

Permeation of plasma fibrin clot was assessed as described [15]. Briefly, tubes containing fibrin clots formed from adding 20 mmol/L calcium chloride and 1 U/mL human thrombin (Sigma) to citrated plasma, were connected through plastic tubing to a reservoir of a buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5). The volume flowing through the gel was measured within 60 min. A permeation coefficient (K_s), which reflects pore size, was calculated from equation: $K_s = Q \times L\ \eta / t \times A \times \Delta p$, where Q is the flow rate in time t, L is the length of a fibrin gel, η is the viscosity of liquid, A is the cross section area and Δp is a differential pressure in dyne/cm^2.

Fibrin clot turbidity

Plasma polymerization was initiated by adding to diluted 1:1 with Tris buffer (0.05 mol/L Tris-HCl, 0.15 M NaCl, pH 7.4) plasma samples 1 U/mL human thrombin (Sigma-Aldrich, St Louis, MO, USA) and 15 mmol/L calcium chloride [21]. Absorbance was read at 405 nm for 15 min with a Perkin-Elmer Lambda 4B spectrophotometer (Molecular Devices Corp., Menlo Park, CA, USA). The lag phase of the turbidity curve, which indicates the time required for initial protofibril formation allowing lateral aggregation and maximum absorbance (ΔAbs), which reflects the fiber thickness, were recorded.

Efficiency of fibrinolysis

Assay 1. Fibrin clot lysis time (CLT) was measured as described [22], with modifications [23]. Briefly, citrated plasma was mixed with 15 mmol/l calcium chloride, 10 000-diluted human TF (Innovin, Dade Behring, Marburg, Germany), 12 μmol/l phospholipid vesicles, and 60 ng/ml recombinant tPA (rtPA, Boehringer Ingelheim, Ingelheim am Rhein, Germany). Turbidity was measured at 405 nm at 37 °C. CLT was defined as the time from the clot formation (the midpoint of the clear-to-maximum-turbid transition) to the clot lysis (midpoint of the maximum-turbid-to-clear transition).

Assay 2. The time required for a 50% decrease in clot turbidity ($t_{50\%}$) was measured as described [24], with modifications [25]. Briefly, 100 μl citrated plasma was diluted 1:1 with Tris buffer (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.4) including 20 mmol/L calcium chloride, 1U/mL human thrombin (Sigma) and 1 μg/mL rtPA. Absorbance was measured at 405 nm.

Assay 3. Fibrin clots formed as in the permeation study was perfused with the same buffer containing 0.2 μmol/l rt-PA as described [16]. D-dimer was measured (American Diagnostica) every 20 minutes in the effluent, which determines the lysis rate. The maximum rate of increase in D-dimer concentration ($D\text{-}D_{rate}$) and maximum D-dimer level ($D\text{-}D_{max}$) detected at 80 or 100 min were analyzed in each subject. The experiment was ended after 80-120 min when the fibrin gel collapsed.

Scanning electron microscopy (SEM)

Clots from randomly selected patients with DTC ($n = 5$) and control subjects ($n = 5$) were fixed by permeating them with a 2.5% glutaraldehyde for 2 hours, recovered from the tubes and further processed by dehydratation as described [15]. Digital photographs of samples were taken in five different areas with a Hitachi S-4700 SEM. Quantitative fibrin network analysis was performed using the Image J software (Bethesda, Maryland, USA). Fiber thickness was measured via generating 100 x/y coordinates at random and then calculating a fiber diameter at or nearest to each of the 100 coordinates only if fiber margins were clearly defined.

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