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Prothrombotic alterations in plasma fibrin clot properties in thyroid disorders and their post-treatment modifications

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ABSTRACT

Introduction: Available data on fibrin clot properties and fibrinolysis in hyperthyroidism and hypothyroidism are inconsistent. Our objective was to assess the impact of effective treatment of hyper- and hypothyroidism on fibrin clot characteristics.

Material and Methods: In a case-control study, *ex vivo* plasma fibrin clot permeability (K_s) and efficiency of fibrinolysis were assessed in 35 consecutive hyperthyroid and 35 hypothyroid subjects versus 30 controls. All measurements were performed before and after 3 months of thyroid function normalizing therapy.

Results: At baseline, hyperthyroid, but not hypothyroid, patients had lower K_s than controls ($p < 0.0001$). Hyperthyroid and hypothyroid groups compared with controls had prolonged clot lysis time (CLT), and lower rate of D-dimer release from clots ($D\text{-}D_{rate}$) (all $p < 0.05$). The regression analysis adjusted for fibrinogen showed that in hyperthyroid patients, pre-treatment thyroid stimulating hormone (TSH) independently predicted K_s, while thrombin activatable fibrinolysis inhibitor (TAFI) antigen predicted CLT. In hypothyroid individuals a similar regression model showed that TSH independently predicts CLT. After 3 months of thyroid function normalizing therapy, 32 (91.4%) hyperthyroid and 30 (85.7%) hypothyroid subjects achieved euthyroidism and had improved fibrin clot properties (all $p < 0.05$), with normalization of K_s in hyperthyroid and lysability in hypothyroid patients.

Conclusions: Both hyper- and mild-to-moderate hypothyroidism are associated with prothrombotic plasma fibrin clot phenotype and restoration of euthyroidism improves clot phenotype. Abnormal fibrin clot phenotype might contribute to thromboembolic risk in thyroid disease.

Introduction

Clinical evidence indicates that hyperthyroidism increases the risk of arterial thromboembolism [1] and unprovoked venous thrombosis [2–4]. Hypothyroidism is also associated with increased venous thromboembolism (VTE) risk [5]. The precise mechanisms underlying hemostatic imbalance in thyroid disorders are not fully elucidated. It has been shown that in overt hypothyroidism, factor VIII and von Willebrand factor (VWF) could be decreased leading to acquired von Willebrand syndrome [6]. α_2-antiplasmin activity, tissue-type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) antigen levels are elevated in mild-to-moderate hypothyroidism, with a tendency to their reduction in severe hypothyroidism [7]. Elevated thrombin-activatable fibrinolysis inhibitor (TAFI) has been reported in both hypothyroidism and hyperthyroidism [8–10]. Increased coagulation factors VIII and IX, VWF, fibrinogen, PAI-1, enhanced platelet aggregability and thrombin generation have been observed in hyperthyroid subjects [11,12]. In both types of thyroid dysfunction, thyroid hormone (especially tri-iodothyronine [T3]) has been reported to be a major factor inducing the hemostatic alterations [3,13].

The final step of blood coagulation is thrombin-mediated conversion of fibrinogen to fibrin. Fibrin clots composed of tightly packed thin fibers with small pores are relatively resistant to lysis [14,15] and such prothrombotic clot phenotype has been shown in patients with myocardial infarction [16,17], ischemic stroke [18], peripheral arterial disease [19], VTE [20], and rheumatoid arthritis [21].

Fibrin clot properties can be altered also in thyroid dysfunction. It has been demonstrated in 19 hyperthyroid patients that plasma clot

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maximum absorbance (a marker of plasma clot density), and lysis area (reflecting clot formation time, clot density, and lysis potential) correlate with free thyroxine (FT4), while exogenous thyroid hormones do not alter those parameters [22]. Using the same high-throughput analysis, it has been reported that maximum clot absorbance is decreased and clot lysis time (CLT) is shorter in 20 hypothyroid subjects with thyroid-stimulating hormone (TSH) levels of 92.3 ± 17.2μIU/ml [23]. In both thyroid disorders, achieving euthyroidism after 2-8 months' therapy was associated with normalized [22] or improved [23] fibrin characteristics (no control group was included in the latter study). It is unclear which factors other than TSH determine altered fibrin clot properties in hyper- and hypothyroidism.

We sought to assess the impact of effective treatment of hyper- and hypothyroidism on plasma fibrin clot properties, using plasma fibrin clot assays, including clot permeability and fibrinolysis efficiency at various tPA concentrations, along with markers of thrombin generation, platelet activation and fibrinolysis.

Material and Methods

Patients

From September 2011 to May 2012, we recruited to a case-control study 35 consecutive newly diagnosed patients with hypothyroidism, and 35 with hyperthyroidism matched for age and sex. All patients were referred to the out-patient clinic at the Department of Endocrinology, Jagiellonian University Medical College, by family physicians for the first time. The diagnoses were established by an experienced clinician. Normal reference ranges used for TSH and FT4 were 0.27-4.20μIU/ml and 12-22 pmol/l. Hypothyroidism was defined as serum TSH above the upper limit of the reference range, i.e. 4.20μIU/ml, and serum FT4 below the lower limit of the reference range, i.e. 12 pmol/l; mild hypothyroidism was with TSH 4.30-10μIU/ml, and moderate with TSH 10-20μIU/ml; if TSH was 4.30-10μIU/ml and FT4 normal, subclinical hypothyroidism was diagnosed. Hyperthyroidism was defined as serum TSH below the lower limit of the reference range, i.e. 0.27μIU/ml, and FT4 above the upper limit of the reference range, i.e. 22 pmol/l; if FT4 was normal, subclinical hyperthyroidism was diagnosed. Graves' disease (GD) was diagnosed in hyperthyroid subjects and the presence of specific human thyroid receptor stimulating antibodies (h-TRAB; concentration >1 IU/l). When specific thyroid antibodies were absent (concentration <1 IU/l) in patients with hyperthyroidism and a palpable nodular goiter and/or typical technetium scintigraphic image were detected, toxic nodular goiter (TNG) was diagnosed. In hypothyroidism, the diagnostic thresholds for anti-thyroid peroxidase antibody (a-TPO) antibodies and anti-thyroglobulin antibody (a-Tg) were 34 IU/ml and 10 IU/ml, respectively. The exclusion criteria were: current anticoagulant or antiplatelet therapy, history of acute coronary syndrome (ACS), stroke or deep vein thrombosis within 6 months prior to enrolment, severe concomitant disease (i.e. malignancy, liver injury with alanine aminotransferase greater than 1.5 times the upper limit of normal, or renal insufficiency with serum creatinine >177 μmol/l), pregnancy, any acute infection and age below 18 years. The age- and sex-matched controls were recruited from the hospital staff and their relatives. All participants gave their written informed consent, and the study was approved by the Jagiellonian University Ethical Committee.

Diabetes mellitus was defined as current treatment with insulin and/or oral hypoglycemic agents, or fasting glycaemia greater than 7 mmol/l measured twice. An individual was classified as having arterial hypertension if he/she met any of the following criteria: (1) previous history of hypertension; (2) current antihypertensive treatment; (3) systolic or diastolic pressure ≥140 mmHg or ≥90 mmHg, respectively, on at least two different occasions. Coronary artery disease (CAD) was defined as a history of ACS, coronary revascularization or known CAD treatment.

Three-month therapy was administered in all patients. Hyperthyroid patients were treated with thiamazole titration (the dose was adjusted fortnightly), or with radioactive iodine therapy (RAI), whereas in hypothyroid patients, levothyroxine (LT4) was administered (dose adjustments were performed monthly).

Laboratory Investigations

Blood was drawn from an antecubital vein with minimal stasis, after an overnight fast, between 8 to 10 AM, prior to the start of therapy and after 3 months (for subjects with thyroid dysfunction), or at one time-point (for controls). Serum glucose, lipid profile and creatinine were assayed by routine laboratory techniques. TSH, FT4, free T3 (FT3), a-TPO and a-Tg were assayed by electrochemiluminescent immunoassays (Roche Diagnostics, Basel, Switzerland). Plasma h-TRAB antibody levels were measured by a radioimmunometric assay (B.R.A.H.M.S, Hennigsdorf, Germany). All measurements were performed by technicians blinded to the sample origin.

Fibrinogen was determined using the Clauss method. High-sensitivity C-reactive protein (hsCRP) was measured by latex nephelometry (Siemens, Marburg, Germany). Lipoprotein (a) (Lp[a]) was measured using an enzyme-linked immunosorbent assay (ELISA) (DRG Diagnostics, Marburg, Germany). Plasma α_2-antiplasmin and plasminogen were measured by chromogenic assays (STA Stachrom α2-antiplasmin and STA Stachrom plasminogen, Diagnostica Stago, Asnières, France). Plasma PAI-1 and tPA antigen levels were measured by ELISAs (Hyphen BioMed, Neuville-Sur-Oise, France), whereas PAI-1 activity by using a chromogenic assay (Chromolize PAI-1, Trinity Biotech, County Wicklow, Ireland). Measurement of TAFI antigen was performed with an ELISA (Chromogenix, Lexington, MA, USA). Plasma TAFI activity was measured by a chromogenic assay using the ACTICHROME® Plasma TAFI Activity Kit (American Diagnostica, Stamford, CT, USA). Plasma platelet activation markers, soluble CD40 ligand (sCD40L) and soluble P-selectin, were assessed by ELISAs (R&D Systems, Minneapolis, MN, USA).

Thrombin Generation

Measurement of the thrombin generation was performed using calibrated automated thrombography (CAT) (Thrombinoscope BV, Maastricht, the Netherlands) according to the manufacturer's instructions in the 96-well plate fluorometer (Ascent Reader, Thermolabsystems OY, Helsinki, Finland), equipped with the 390/460 filter set, at a temperature of 37 °C. Briefly, 80 microliters of platelet-poor plasma were diluted with 20 μL of the reagent containing 5 pmol/l recombinant tissue factor, 4 micro-molar phosphatidylserine/phosphatidylcholine/phosphatidylethanolamine vesicles, and 20 μL of FluCa solution (Hepes, pH 7.35, 100 nmol/l $CaCl_2$, 60 mg/ml bovine albumin, and 2.5 mmol/l Z-Gly-Gly-Arg-7-amino-4-methylcoumarin). Each plasma sample was analyzed in duplicate. For analysis, the maximum concentration of thrombin generated was used.

Fibrin Clot Analysis

Plasma fibrin clot variables were determined at least in duplicate (for all variables intraassay and interassay coefficients of variation were 5% to 8%). Plasma fibrin clot permeability was determined in a hydrostatic pressure system, as described [20]. 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 buffer reservoir (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), reflecting pore size, was calculated from equation: $K_s = Q \times L \times \eta / t \times A \times \Delta p$, 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. Lower K_s values indicate reduced permeability.

Fibrinolysis induced by recombinant tPA (rtPA, Boerhinger Ingelheim, Ingelheim, Germany), expressed as CLT, was measured