Thrombosis Research 139 (2016) 56–64

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

NETosis promotes cancer-associated arterial microthrombosis presenting as ischemic stroke with troponin elevation

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ARTICLE INFO

Article history:
Received 16 October 2015
Received in revised form 23 December 2015
Accepted 10 January 2016
Available online 12 January 2016

Keywords:
Cancer-associated microvascular thrombosis
Neutrophil extracellular traps
Troponin elevation
Ischemic stroke

ABSTRACT

Introduction: Large elevations of high sensitive Troponin T (hsTnT) in ischemic stroke patients is associated with a poor outcome. In a pilot study we found a high prevalence of malignancies among these patients. Since neutrophil extracellular traps (NETs) have been linked to cancer-associated thrombosis, we hypothesized that the concomitant cerebral and myocardial ischemia could be the result of a NET-induced hypercoagulable state.
Materials and methods: Clinical assessments, plasma analyses and autopsies with histopathology (in cases of in-hospital mortality) were performed on ischemic stroke patients with high elevations of hsTnT ($N = 12$) and normal hsTnT ($N = 19$).
Results: Patients with hsTnT elevation had an unexpectedly higher prevalence of cancer ($p = 0.002$), half of which were diagnosed post-mortem. Autopsies of these patients revealed widespread myocardial, cerebral and pulmonary microthrombosis with H3Cit in thrombi. A pro-coagulant state and an increase of the NET specific marker citrullinated histone *H*3 (H3Cit) was found in plasma of patients with elevated hsTnT compared to patients with normal levels ($p < 0.001$). Plasma analyses in cancer patients showed even higher H3Cit levels ($p < 0.001$), and an increase in granulocyte colony-stimulating factor, known to prime neutrophils towards NETosis. H3Cit correlated positively with thrombin-antithrombin complex ($p = 0.004$) and soluble *P*-selectin ($p < 0.001$), further linking NETosis to the pro-thrombotic state.
Conclusions: The high prevalence of known or occult cancer in our study suggests that cancer-associated arterial microthrombosis may be underestimated. By linking the thrombosis to NETs, we suggest markers of NETosis that could aid in revealing cancer in arterial microthrombosis as well as arterial microthrombosis in cancer.
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1. Introduction

Cerebrovascular and cardiovascular thrombosis is a leading cause of death. Ischemic stroke can be associated with a variety of cardiac changes, including plasma elevations of cardiac enzymes such as troponins. Previous studies have shown that troponin elevation in ischemic stroke is associated with an overall increased risk of poor outcome and mortality [1]. The underlying pathophysiology of troponin elevation in ischemic stroke is still unclear. A number of possible mechanisms have however been proposed, such as concomitant acute coronary syndrome (ACS) [2], neurologically induced myocardial injury due to sympathoadrenal activation [3], atrial fibrillation, congestive heart failure (CHF), renal insufficiency and severe infection [4,5].

There is now growing evidence of neutrophil extracellular trap (NET) burden in a variety of thrombotic diseases: among them ischemic stroke [6], ACS [7,8] and cancer-associated thrombosis [9–11]. NETs were first described in 2004 [12] as a mechanism for trapping and killing of bacteria by the innate immune system. Upon activation, neutrophils release chromatin (DNA and histones) coated with antimicrobial granular proteins such as myeloperoxidase (MPO). Prior to releasing NETs, the protein citrullinating enzyme peptidylarginine deiminase 4

Abbreviations: ACS, acute coronary syndrome; CHF, congestive heart failure; NETs, neutrophil extracellular traps; PAD4, peptidylarginine deiminase 4; H3Cit, citrullinated histone *H*3; hsTnT, high sensitivity Troponin T; G-CSF, granulocyte colony-stimulating factor; NIHSS, National Institute of Health Stroke Scale; TAT, thrombin-antithrombin complex; sP-selectin, soluble *P*-selectin; cfDNA, cell free DNA; VTE, venous thromboembolism; LMWH, low-molecular-weight heparin.

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http://dx.doi.org/10.1016/j.thromres.2016.01.009
0049-3848/Published by Elsevier Ltd.

(PAD4) enters the nucleus and citrullinates histones initiating chromatin decondensation. Citrullinated histone *H*3 (H3Cit) is thereby considered a NET specific marker. NETs have been found to promote coagulation by activation of platelets and coagulation factors as well as providing a scaffold for platelets and red blood cells, promoting thrombus formation [13,14].

In an attempt to better understand the mechanisms leading to troponin elevation in ischemic stroke patients, a pilot-study was conducted and ischemic stroke patients were selected in a case-control design on the basis of highly elevated or normal levels of plasma high sensitivity Troponin T (hsTnT). The presence of comorbidities, such as ACS, CHF, atrial fibrillation and renal insufficiency, did not differ between the groups with and without troponin elevation, ruling out the implication of these previously suggested mechanisms. Instead, we found an unexpectedly high prevalence of malignancies among the patients with large hsTnT-elevations. Autopsy and histopathological investigation performed on three patients with elevated hsTnT and malignancies revealed widespread arterial H3Cit-positive microthrombosis. We hypothesized that high elevations of plasma troponin in ischemic stroke patients could be the result of a cancer-associated NET-induced procoagulant state leading to concomitant cerebral and myocardial ischemia. In light of a recent report on cancer-associated granulocyte colony-stimulating factor (G-CSF) priming neutrophils towards NETosis in mouse models [9], we sought to examine the contribution of circulating G-CSF in NET formation. We report elevated levels of circulating G-CSF in these patients, as well as positive correlations between circulating G-CSF, markers of coagulation, and NETs, linking a cancer-induced systemic NET burden to widespread arterial microthrombosis presenting as stroke with troponin elevation.

2. Methods

2.1. Study population and design

A prospective, observational case-control study including 31 patients with ischemic stroke admitted to the stroke unit at Danderyd Hospital, Stockholm, between April 2012 and December 2014. Patients with ischemic stroke and hsTnT > 40 ng/L (ref value < 15 ng/L) were recruited as case patients (N = 12) and patients with ischemic stroke and hsTnT ≤ 15 ng/L were recruited as control patients (N = 19). They were matched according to sex and age within a five-year interval. Inclusion criteria for both groups were 1) ischemic stroke confirmed by cerebral imaging or ischemic stroke with new focal neurological deficits and 2) symptom onset < 48 h before admission. Exclusion criteria for both groups were acute cardiovascular event (ACS or ischemic stroke) within four weeks of symptom onset. Plasma concentration of hsTnT was analyzed on admission using the ECLIA electrochemiluminescence immunoassay system (Roche Diagnostics Scandinavia AB). The assay performance for the cut-off values (≤15 ng/L for normal values and >40 ng/L for elevation) is in accordance with current guidelines. Inclusion was restricted to time periods with available research personnel, during which ischemic stroke patients with the highest plasma hsTnT value on admission were selected to the case group. Healthy volunteers (N = 10), matched on sex and age within a five-year interval, were recruited as reference for plasma analyses.

Demographic data and comorbidity were obtained from medical records and patient history documented on admission. Active cancer was defined as diagnosis of, or treatment for, cancer within the prior six months, known recurrent or metastatic disease, or diagnosis within two months after stroke onset.

CT brain imaging was performed on admission and stroke localization and distribution was determined by a senior neuroradiologist blinded to clinical details. Stroke severity was determined using the National Institute of Health Stroke Scale (NIHSS) by certified raters.

2.2. Plasma analyses

Blood samples were drawn within two days of admission with patients in bed in the supine position 30 min prior to blood sampling. Plasma samples were prepared from citrated whole blood following immediate centrifugation for 20 min at 2000 *g* after which they were stored at −80 °C until further analyses. Plasma analyses were performed at the Wagner Laboratory, Boston Children's Hospital. At time of analyses, samples were thawed once. Thrombin-antithrombin complex (TAT), soluble *P*-selectin (sP-selectin), cell free DNA (cfDNA), G-CSF and MPO were analyzed with human TAT ELISA (Enzygnost TAT mikro, Siemens), human sP-selectin/CD62P Quantikine ELISA (R&D Systems), Quant-iT PicoGreen dsDNA assay (Invitrogen), human G-CSF Quantikine ELISA (R&D Systems) and human myeloperoxidase Quantikine ELISA kit (R&D Systems), according to the manufacturer's instructions. H3Cit was detected using a tailor-made capture ELISA method. Briefly, the microplate modules of the cell death detection ELISA were coated with the anti-histone antibody (Component 1) overnight and blocked with the incubation buffer (Roche). Plasma samples were incubated for 1 h, washed with PBS-Tween 0.05%, followed by incubation with rabbit anti-histone *H*3 (citrulline 2 + 8 + 17) antibodies (Abcam) and anti-rabbit-horseradish peroxidase-conjugated antibodies (Biorad). The samples were analyzed side-by-side on the same plate, and the inter-individual differences in duplicates were negligible (mean difference: 0.007 O.D.).

2.3. Autopsy and histopathology

Specimens obtained at autopsy were stained with standard hematoxylin & eosin, Luxol fast blue for degenerated neural tissue and Ladewigs trichrome for fibrin. Immunohistochemistry was performed on specimens containing thrombi. The antibody used was anti-histone *H*3 (citrulline 2 + 8 + 17) antibody (Abcam) for H3Cit. Confocal immunofluorescence microscopy at the Wagner Laboratory, Boston Children's Hospital, was performed with antigen retrieval in sodium citrate buffer (10 mM, pH 6.0) using microwave after deparaffinization. The sections were permeabilized with 0.1% Triton X-100 on ice for 10 min. After blocking with 3% bovine serum albumin (BSA) for 1 h at 37 °C, slides were incubated overnight at 4 °C with sheep polyclonal anti-von Willebrand factor (anti-VWF, Abcam, ab11713, 1:250), mouse monoclonal anti-human smooth muscle actin (anti-SMA, Dako, M0851, 1:100) and rabbit polyclonal anti-H3Cit (Abcam, ab5103, 1:1000) in antibody dilution buffer (0.3% BSA, 0.05% Tween-20) and then with Alexa Fluor-conjugated secondary antibodies (Invitrogen, 1:1500) for 2 h at room temperature after washes in phosphate-buffered saline. DNA was stained with Hoechst 33342 (1:10,000). Images were acquired with Olympus Fluoview software using the Olympus IX 81 confocal microscope.

The study complied with the Declaration of Helsinki and the protocol was approved by the Stockholm local ethics committee (dnr 2011/1310-31/3 and 2014/442-31/4). Written informed consent was obtained from each study participant or a family member.

2.4. Statistics

Statistical methods were chosen to fit small numbers of observations and non-normal distributions. Categorical variables are presented as proportions and compared with the Fisher's exact test. Continuous variables are presented as medians with interquartile ranges (IQR) and compared with the Mann-Whitney *U* test. Significance of correlation was analyzed with Spearman's rank correlation. All statistical analyses were performed using STATA 12.1 software (STATA, Texas, USA) and a p-value < 0.05 was considered statistically significant.

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