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Full Length Article

Thrombin generation and intracranial atherosclerotic disease in patients with a transient ischaemic attack

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ABSTRACT

Background: Intracranial atherosclerotic disease (ICAD) is responsible for at least 10% of transient ischaemic attacks (TIA). Thrombin generation has been shown to be associated with several atherosclerotic conditions and may be relevant in the pathogenesis of TIA from ICAD.
Objective: To evaluate the association between thrombin generation and ICAD in patients with TIA.
Materials and methods: Consecutive patients with confirmed diagnosis of TIA by vascular neurologist were enrolled. Within 24 h from diagnosis, all the patients underwent: blood samples including thrombin generation search, electrocardiography, brain CT scan, blood pressure (BP) measurement, supra-aortic echo-Doppler, transcranial Doppler (TCD) and standard echocardiogram. Thrombin generation was measured as endogenous thrombin potential (ETP) in platelet-rich plasma (PRP) and in platelet-poor plasma (PPP), in the presence and in the absence of thrombomodulin (TM).
Results: 120 patients (male 52.5%), aged 69 ± 16 years were enrolled. Ten patients on warfarin treatment had significantly lower ETP than the others. Among the remaining, ETP in the presence or absence of TM did not differ according to TOAST classification aetiology (large vessel vs. cardioembolic vs. lacunar vs. others). In PRP, ETP was similar in patients with ICAD and in those without (1748 ± 160 vs. 1851 ± 36 nM·min, $p = 0.393$), whereas, ETP measured in presence of thrombomodulin was higher in patients with than in those without ICAD (2045 ± 99 vs. 1715 ± 41 nM·min, $p = 0.011$). In PPP, ETP was similar in patients with ICAD and in those without, whereas thrombin peak was higher in patients with ICAD than in those without both in the presence (165 ± 17 vs. 130 ± 5 nM, $p = 0.036$) and in the absence of TM (178 ± 19 vs. 142 ± 5 nM, $p = 0.037$).
Conclusion: ETP measured in presence of TM is enhanced in patients with ICAD, supporting that thrombomodulin-protein C pathways is relevant in TIA from ICAD. These hypothesis-generating data suggest that thrombin generation may be relevant in cerebral ischaemia from intracranial disease, and justify larger studies.

1. Introduction

Recurrent stroke is the major cause of morbidity and mortality among patients with transient ischaemic attacks (TIA) [1]. Because the risk of stroke is highest in the first days after stroke, the identification of a possible mechanism of early recurrence is of great importance to develop effective treatments for secondary stroke prevention. Intracranial atherosclerotic disease (ICAD) is responsible for approximately 10% of ischaemic strokes/TIA in non-Asian patients [2], and in patients who have experienced a minor stroke or TIA, ICAD is associated with a stroke recurrence of 15% [3]. Activation of platelets and coagulation system is an important step in the early phases of TIA [4] and hemostatic activation with increased coagulation and platelet activation may be a possible mechanism for TIA and for early stroke recurrence in patients with ICAD. Thrombin generation, measured as the potential to form thrombin has been may be linked to risk of atherothrombosis (coronary or cerebral) [5]. A prospective cohort study showed that increased thrombin generation was an independent predictor of ischaemic stroke, especially cardioembolic stroke [6]. In patients with acute stroke/TIA, enhanced thrombin formation has been demonstrated [7] and patients with recently symptomatic carotid stenosis have higher thrombin generation potential than patients with asymptomatic carotid stenosis [8]. To our knowledge, no data have been reported about the relationship between TIA from ICAD and activation of coagulation system. Since ICAD is often associated with in situ thrombotic occlusion, artery-to-artery embolism, and branch occlusion [9–10], we speculated an increased coagulation activation in acute phase of TIA from ICAD. Thus, aim of the present study was to evaluate the association between

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thrombin generation measured in first hours of TIA and the presence of ICAD.

2. Methods

2.1. Study population

This was a cross-sectional study on Caucasian patients with acute TIA. Consecutive patients were recruited from the Emergency Department admission from August 1, 2010, to July 31, 2013 to Thrombolysis Center and Stroke Unit of S. Orsola-Malpighi University Hospital (Bologna, Northern Italy). TIA definition was a sudden, focal neurological deficit lasting <24 h, presumed to be of vascular origin, and confined to an area of the brain or eye perfused by a specific artery [11]. Exclusion criteria were: active cancer and severe dementia. As described elsewhere [12], the first-line work-up included immediate neurological evaluation, electrocardiography, brain CT scan, blood samples, blood pressure (BP) measurement, supra-aortic echo-Doppler, transcranial Doppler (TCD) and standard echocardiogram within 24 h. ABCD2 score was also recorded. A second-level assessment included standard and transesophageal echocardiogram, prolonged ECG and BP monitoring, CT or MR-angiography. All TIA were validated by a senior vascular neurologist using the TOAST classification for stroke [13] which distinguishes cerebral ischaemic events in large vessels atherothrombotic (LVA), cardioembolic (CE), small vessels disease (SVD), acute ischaemic events of other aetiology, and acute ischaemic events of unknown cause (because of incomplete workup, or of more than one likely aetiology, because a single most likely aetiology cannot be determined, or no probable aetiology determined despite complete workup) [13]. In our analysis, we divided TIA into four groups: LVA, SVD, CE and all other aetiologies or unknown cause. Data were collected regarding vascular risk factors, including hypertension, prior TIA or stroke, ischaemic heart disease, atrial fibrillation, valvular heart disease, diabetes mellitus, hyperlipidaemia, atrial fibrillation, family history of stroke, medication intake (including antithrombotic therapy), smoking status. Accordingly, each risk factor was coded as either present or absent.

As control subjects, 46 healthy individuals (male/female 21/25) aged 52.5 ± 11.3 y (range 21–71) were enrolled in this study.

All subjects gave informed consent. The study complied with the Declaration of Helsinki and was approved by the local ethics committee.

2.2. Transcranial duplex ultrasound examination

ICAD was defined as a stenosis >50% or occlusion of the intracranial arteries on transcranial duplex ultrasound (TCD). All patients underwent a TCD that was performed by a senior vascular physician according to the protocol of Meseguer E. et al. [14]. Briefly, TCD examination included middle cerebral artery (MCA), carotid siphon (CA), vertebral artery (VA) on both sides, and basilar arteries (BA). Stenosis >50% was defined as a peak systolic velocity increase >150 cm/s for proximal MCA, >120 cm/s for VA or BA, and >100 cm/s for CA or a difference >30% compared with the contralateral artery. MCA occlusion was diagnosed if all other basal arteries were detectable or if the asymmetry index of the symptomatic MCA was <−21% compared with that of the contralateral MCA. CA occlusion was diagnosed if all basal arteries except the CA and the ipsilateral MCA were not detected. BA occlusion was diagnosed if there was a high-resistance flow pattern at depths of 85 to 95 mm in the BA, possibly combined with a sudden loss of flow signals when increasing the examination depth, or retrograde flow in the distal BA. Intracranial VA occlusion was diagnosed if there was a resistance flow profile in one side whereas normal or even compensatory elevated flow velocity was seen on the other side. All patients with TCD stenosis/occlusion were examined with another form of cerebral vascular imaging (magnetic resonance angiography or computed tomography angiography) to confirm ICAD.

2.3. Blood sampling and thrombin generation assay

Blood was collected from the antecubital vein into 0.109 mmol/L trisodium citrate within 24 h from TIA; platelet-poor and platelet-rich plasmas (PPP and PRP, respectively) were prepared by centrifugation for 20 min at 2000 × *g* and 10 min at 200 × *g* at controlled room temperature, respectively. Platelet count in PRPs was carried out by a haematology analyzer (Sysmex K-4500, Dasit, Cornaredo, Milan, Italy).

The thrombin generation assay was performed in fresh PRP and PPP using the Technothrombin TGA kit (Technoclone, Wien, Austria). The test is based on monitoring the fluorescence generated by thrombin cleavage of a fluorogenic substrate over time upon activation of the coagulation cascade by different concentrations of tissue factor and negatively charged phospholipids according to a method described elsewhere [15]. In our study the test was carried out using low concentration of phospholipid micelles containing ≈5 pM tissue factor in Tris-HEPES-NaCl buffer. Tests were repeated in a second aliquot of plasma by adding soluble thrombomodulin (4 nM final concentration; ICN Biochemicals, Aurora, OH, USA). The test was performed using an automated analyzer (Ceveron Alpha, Technoclone, Wien, Austria); thrombin generation is measured with a special adapted TGA fluorimetric module, which is placed over the cuvette rotor, with an UV emitter (360 nm) placed in the module. Calibration was performed using a thrombin calibrator provided with the kit (≈1.000 nM thrombin in buffer with BSA). Readings from the fluorometer are automatically recorded and elaborated by a software which displays thrombin generation curves [nM thrombin vs. time (min)]; the time of lag-phase that follows the addition of the trigger (min), the thrombin peak (nM), the time to peak (min) and the area under the curve defined as endogenous thrombin potential (ETP, nM thrombin·min) are then calculated. ETP levels for patients and healthy subjects were used to calculate the ratios between the values obtained with and without thrombomodulin.

Operators testing for thrombin generation were unaware of the patients' characteristics.

2.4. Statistical analysis

Analysis was carried out using the SPSS™ software package (version 21; IBM Corp., USA). Relations between variables were assessed using Pearson correlation for continuous variables and chi square or Fisher exact test for categorical variables. Student's *t*-test and multivariate analysis of variance with Bonferroni's correction for multiple comparisons were used to compare means among groups. Analysis of covariance (ANCOVA) was used for the variable ETP for comparison of ICAD patients with adjustment for platelet number. The variable ETP was also dichotomized with the upper tertile value as the cut-off point. The associations between clinical characteristics of the study population and the presence of ICAD were tested with univariable analysis followed by multivariable analysis. A parsimony model with predictors associated with a *p*-value >0.1 was presented to improve precision and avoid over-fitting. The significance level was set at <0.05. Values are expressed as means ± SEM.

3. Results

From August 1, 2010, to July 31, 2013, we enrolled 120 patients with confirmed diagnosis by a vascular neurologist. Baseline patient characteristics are reported in Table 1. At enrollment, ten patients were receiving vitamin K antagonists: as expected [16], they had significantly lower ETP than the other patients either in the absence (1097 ± 182 vs. 1840 ± 36, $p = 0.001$) or in the presence of thrombomodulin (928 ± 165 vs. 1748 ± 39, $p = 0.001$) and consequently they were excluded from the subsequent analysis. Among the remaining 110 patients, aetiology was defined as LVA in 30 patients (27.3%), SVD in 25 patients (22.7%), CE in 21 (20.0%), and cryptogenetic or other aetiology in 34 patients (30%) (Table 2). As reported in Table 2, ETP, measured in the absence or