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Plasma endotoxin activity rises during ischemic stroke and is associated with worse short-term outcome

Elzbieta Klimiec, Joanna Pera, Joanna Chrzanowska-Wasko, Aleksandra Golenia, Agnieszka Slowik, Tomasz Dziedzic *

Department of Neurology, Jagiellonian University Medical College, Krakow, Poland

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ABSTRACT

Background: Activation of Toll-like receptor 4 (TLR4) contributes to brain injury and poor outcome after cerebral ischemia. The expression of this receptor on monocytes is increased in patients with acute ischemic stroke. Endotoxin is an endogenous ligand for TLR4. The aim of our study was to determine if plasma endotoxin activity is increased in stroke patients and correlates with functional outcome.
Methods: We included 88 patients with ischemic stroke (median age: 71, 56.8% men) and 59 age-matched controls. Plasma endotoxin activity and level of proteins regulating endotoxin interaction with TLR4 (LPS binding protein – LBP and sCD14) were measured in blood samples taken at day 1 (within 24 h after stroke symptoms onset), 3 and 6. Short-term functional outcome was assessed at day 14 using modified Rankin Scale. Unfavourable outcome was defined as modified Rankin Scale score > 2.
Results: Compared to controls, stroke patients had higher plasma endotoxin activity on day 1 (median: 0.39 vs 0.32 EU/mL, $P = 0.03$) as well as higher LBP (median: 18.7 vs 11.5 μg/mL, $P < 0.01$) and sCD14 level (median: 1330 vs 1070 ng/mL, $P < 0.01$). Plasma LPS activity and levels of LBP and sCD14 significantly rose during stroke. Higher LPS activity measured on day 6 was associated with unfavourable outcome (OR: 3.94, 95%CI: 1.03–15.02, $P = 0.04$, adjusted for age and stroke severity).
Conclusions: Plasma endotoxin activity rises during ischemic stroke and is associated with worse short-term outcome.

1. Introduction

Toll-like receptors (TLRs) are key regulators of innate immunity and play an important role in brain damage after ischemic stroke (Gesuete et al., 2014). TLR4 is one of the most extensively studied TLRs in ischemic brain injury. Compared to wild-type mice, TLR4-deficient mice have smaller infarct volume, better behavioural outcome and diminished inflammatory response after cerebral ischemia (Caso et al., 2007; Tang et al., 2007). In patients with cerebral infarction, TLR4 expression on monocytes increases during stroke and correlates with circulating inflammatory mediators (tumor necrosis factor alpha, interleukin-1 beta and interleukin-6) and stroke severity (Yang et al., 2008; Brea et al., 2011). In addition, increased expression of TLR4 on monocytes is independently associated with poor functional outcome after stroke (Brea et al., 2011).

Endotoxin (lipopolysaccharide, LPS) is a glycolipid located in the outer membrane of Gram-negative bacteria. LPS is a main agonist of TLR4 and a potent immunostimulator. LPS binding protein (LBP) and CD14 play a key role in endotoxin signaling (Kitchens and Thompson, 2005; Lu et al., 2008). LBP is a soluble protein which binds to LPS and deliver it to CD14 resulting in target cell activation. LBP is produced by gastrointestinal and hepatic epithelial cells. CD14 is a pattern-recognition receptor which also exists in soluble form (sCD14) secreted by monocytes and macrophages. CD14 facilitates the transfer of LPS to TLR4/MD-2 complex. Naturally occurring immunoglobulin antibodies to the LPS core oligosaccharide can neutralize LPS activity (Cohen and Norins, 1966).

Endotoxin can be detected in plasma even in apparently healthy individuals. In the Bruneck Study, the median plasma endotoxin level in healthy volunteers without clinical evidence of infections was 14.3 pg/mL (range: 6–209 pg/mL) (Wiedermann et al., 1999). Levels of endotoxin in this range are able to induce inflammatory response in human monocytes (Stoll et al., 2004). Circulating endotoxin may derive from Gram-negative bacteria colonized gastrointestinal, genitourinary and respiratory tracts.

Yang et al. found that serum heat shock protein (HSP)60, an endogenous agonist of TLR4, is increased in patients with ischemic stroke or transient ischemic attack (Yang et al., 2008). In another study, serum HSP70 measured on admission correlated with TLR4 expression on

* Corresponding author at: Department of Neurology; Jagiellonian University Medical College, 31-503 Kraków, ul. Botaniczna 3, Poland.
E-mail address: Dziedzic@cm-uj.krakow.pl (T. Dziedzic).

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monocytes at day 3 and day 7 after stroke (Brea et al., 2011). In this study, we hypothesized that plasma activity of endotoxin, a main ligand for TLR4, is increased in stroke patients and correlates with worse short-term outcome.

2. Materials and methods

The participants for this study were recruited from stroke patients hospitalized in Department of Neurology, Jagiellonian University, Krakow, Poland. The inclusion criteria were: (1) ischemic stroke; (2) time from stroke symptoms onset to admission <24 h; (3) pre-stroke modified Rankin Scale (mRS) 0–2. Exclusion criteria were (1) a lack of patient's informed consent and (2) history of autoimmune or chronic inflammatory diseases.

The study protocol was approved by Bioethics Committee of Jagiellonian University (KBET/265/B/2011). Each participant gave informed consent.

Stroke severity on admission was assessed using the National Institute of Health Stroke Scale (NIHSS). Stroke etiology was defined using TOAST criteria (Adams et al., 1993). Functional outcome was assessed at day 14. Unfavourable outcome was defined as mRS 3–6 and favourable outcome as mRS 0–2. The diagnosis of pneumonia required the presence of clinical findings (at least 2 from the following: productive cough and/or purulent sputum; rales; fever; leukocytosis) and pulmonary infiltrates in chest X-ray. Symptomatic urinary tract infection was diagnosed when (1) at least 1 of following signs: fever (>38 °C), urgency, dysuria, or suprapubic tenderness and positive urine culture (>= 10^5 microorganisms per cc with no >2 species of microorganisms) or (2) at least 1 of following signs: fever (>38 °C), urgency, dysuria, or suprapubic tenderness and at least 2 of the following: positive dipstick for leukocyte esterase and/or nitrate; pyuria (urine specimen with >= 10 white blood cell/mm^3); organisms seen on Gram's stain of unspun urine. Taking into account that asymptomatic urinary tract infections could be a source of circulation endotoxin and that it is impossible to obtain information about urinary symptoms in some stroke patients (patients with aphasia or disturbed consciousness), we decided to broaden the criteria of urinary tract infection. For purpose of this study, we also diagnosed urinary tract infection in patients without symptoms when (1) positive urine culture (>= 10^5 microorganisms per cc with no >2 species of microorganisms) or at least 2 of the following: positive dipstick for leukocyte esterase and/or nitrate; pyuria (urine specimen with >= 10 white blood cell/mm^3); organisms seen on Gram's stain of unspun urine.

Blood samples were taken at day 1 (within 24 h after stroke symptoms onset), 3 and 6. Plasma LPS activity was measured using QCL-1000 Endpoint Chromogenic LAL Assay (Lonza, Walkersville, MD). Before assay, the samples were heated at 70 °C for 10 min to inactivate plasma proteins. The detection limit for this assay was 0.1 EU/mL. Commercially available ELISA kits from Hycult Biotech (Netherlands) and R&D Systems (Minneapolis, MN) were used to measure plasma LBP and sCD14 levels, respectively. Naturally occurring IgG neutralizing antibodies against LPS (EndoCAb) were quantified using ELISA kit from Hycult Biotech (Netherlands).

Mann-Whitney's test was to compare continuous variables between 2 groups and Friedman ANOVA test to compare repeated measurements. Kruskal-Wallis test was used to compare 3 groups (stroke patients with infection, stroke patients without infection and control group). Logistic regression was used to determine the predictors of unfavourable outcome. Since LPS activity did not fulfil the linearity assumption of an interval-dependent variable, in logistic regression analysis we used values dichotomized according the median instead of continuous variable. The calculations were performed using the program STATISTICA for Windows (version 10, Statsoft, Poland). The results are shown as a median with interquartiles.

Table 1
Baseline characteristics of stroke patients and control group.

	Stroke patients (N = 88)	Control group (N = 59)	P
Age, median (interquartiles)	71.0 (62.0–77.5)	69.0 (61.0–77.0)	0.63
Male, n (%)	50 (56.8)	25 (42.4)	0.09
Hypertension, n (%)	66 (75.0)	16 (27.1)	<0.01
Diabetes mellitus, n (%)	25 (28.4)	4 (6.8)	<0.01
Atrial fibrillation, n (%)	28 (31.8)	1 (1.7)	<0.01
Myocardial infarction, n (%)	20 (22.7)	3 (5.1)	<0.01
NIHSS score on admission, median (interquartiles)	11.0 (5.5–18.0)	–	–
Stroke subtype			
TACI, n (%)	33 (37.5)	–	–
PACI, n (%)	32 (36.4)		
LACI, n (%)	19 (21.6)		
POCI, n (%)	4 (4.5)		
Stroke etiology			
Large vessel disease, n (%)	15 (17.0)	–	–
Small vessel disease, n (%)	10 (11.4)		
Cardioembolic, n (%)	27 (30.7)		
Undetermined, n (%)	36 (40.9)		
In-hospital infection, n (%)	45 (51.1)		
Pneumonia, n (%)	25 (28.4)		
Urinary tract infection, n (%)	30 (34.1)		

NIHSS: National Institute of Health Stroke Scale; TACI: total anterior circulation infarct; PACI: partial anterior circulation infarct; LACI: lacunar infarct; POCI: posterior circulation infarct.

3. Results

From 100 consecutive ischemic stroke patients, 94 patients fulfilled inclusion criteria. We excluded 5 patients who did not give consent for participation in the study and 1 patient who was treated with steroids due to rheumatoid arthritis. Our final cohort included 88 stroke patients. The baseline characteristics of stroke cohort and control group are shown in Table 1.

Compared to 59 age-matched controls without stroke (age: 69.0 [61.0–77.0]; male: 42.4%), stroke patients had higher plasma LPS activity on day 1 (0.39 [0.26–0.52] vs 0.32 [0.16–0.46] EU/mL, P = 0.03). Plasma LBP (18.7 [16.2–25.5] vs 11.5 [9.2–14.5] μg/mL, P < 0.01) and sCD14 levels (1330 [1140–1500] vs 1070 [921–1220] ng/mL, P < 0.01) measured on day 1 of stroke were higher than in control group. EndoCAb level did not differ between groups (71.0 [43.0–137.0] vs 79.0 [58.5–103.5] EU/mL, P = 0.80).

Serial measurements of studied parameters were performed in 86 stroke patients. Plasma LPS activity as well as concentration of LBP and sCD14 significantly rose during stroke (Figs. 1–3).

Plasma LBP and sCD14 level measured on day 3 and 6 were higher in patients with infection compared to those without infection (Table 2). Plasma LPS activity did not differ between these two groups of patients. The same trend was observed when the results were analysed separately for patients with pneumonia and patients with urinary tract infections.

Non-parametric ANOVA and post-hoc test for 3 groups (stroke patients with infection, stroke patients without infection and control group) revealed that LPS activity at day 1 did not differ between controls and patients with infection (P = 0.18) as well as between controls and patients without infection (P = 0.19).

Fifty-seven percent of patients had unfavourable outcome at day 14. On univariate logistic regression analysis, higher LPS activity measured on day 6 (OR: 2.71, 95%CI: 1.11–6.57, P = 0.02), but not on day 1 (OR: 1.20, 95%CI: 0.51–2.83, P = 0.67), predicted worse functional outcome. The association between LPS activity measured on day 6 and outcome remained significant after adjusting for NIHSS score on admission and