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Introduction

Ischemic stroke was consistently reported to result in the elevation of brain-derived neurotrophic factor (BDNF), a crucial actor in synaptic and vascular remodeling, either in ischemic or non-ischemic regions in animals [1-3]. Moreover, systemic or local administration of BDNF ameliorates functional motor recovery in ischemic stroke models [4, 5]. Assuming that circulating BDNF levels mirror brain levels, measurement of serum BDNF levels in the early phase of stroke was logically expected to be useful for outcome prediction in patients. However, conflicting data were obtained. While certain studies reported an association between low serum BDNF levels in the acute stroke period and poor short term (7 days to 3 months) outcome [6, 7], other did not [8, 9]. In the blood, BDNF is not only present in platelets, from which it is secreted in response to coagulation process, but also in lymphocytes and monocytes [10, 11]. As these peripheral blood mononuclear cells (PBMC) infiltrate the ischemic brain early and sustainably [12], they represent a potential source of BDNF for the ischemic tissue and could consequently favorably influence stroke outcome. Therefore, the present study investigated the hypothesis that BDNF present in PBMC may influence outcome in ischemic stroke patients. As the infiltration of ischemic tissue by PBMC requires blood supply to the ischemic regions, the study was conducted in patients treated with tissue-plasminogen activator (t-PA) within the 4.5 h following stroke onset.

Methods

Study patients

A cross-sectional observational study was conducted at Dijon University Hospital from January to September 2017 as part of PARADISE study (Prognosis After Revascularization therApy in the Dijon Ischemic Stroke Evaluation study). The PARADISE study received a

favorable opinion from the Committee for the Protection of People (N ° CPP EST I: 2015/32). Patients were admitted in neurovascular intensive care unit for ischemic stroke. All received t-PA within the 4.5 h following stroke onset. Informed consent was given by the patients themselves or by a close relative.

Baseline informations

At hospital admission, demographic data (age, sex), body mass index and conventional cardiovascular risk factors such as hypertension (defined as use of hypertensive medication or systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg at discharge), diabetes (defined as use of glucose-lowering medication or HbA1c ≥6.5%), tobacco consumption (current or ex-smoker), hypercholesterolemia (defined as use of cholesterol-lowering medication, fasting low-density lipoprotein cholesterol >160 mg/dL, or fasting total cholesterol >240 mg/dL) were listed. The cardiovascular health status was assessed by using the European Society of Cardiology (ESC) scale taking account of gender, age, total cholesterol, systolic blood pressure measured at admission and smoking status. Stroke severity was assessed at admission, at day 1 after admission and at hospital discharge using NIHSS (National Institute of Health Stroke Score)**.** The etiology of stroke was classified using TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria. Non-inclusion criteria were hemorrhagic stroke, patients benefiting from thrombectomy, transient ischemic attack, severe infection and HIV (defined by questioning the patient himself or a close relative), prior dementia (defined by use of donepezil, galantamine, rivastigmine or memantine), depression (defined as use of antidepressant medication) and psychiatric illness (defined as use of antipsychotic medication).

Preparation of samples and analysis of BDNF

Venous blood was collected in 2 tubes (an EDTA tube and a gel & clot activator tube for PBMC and serum preparation, respectively) at hospital admission before (day 0, D0) and after (D1 and D3) fibrinolysis for determination of complete blood count (D0) and measurement of BDNF levels in serum and PBMC (D0, D1 and D3). PBMC that were isolated from blood using Ficoll-Hypaque (Eurobio, Courtaboeuf, France) gradient method, were then washed twice with phosphate buffer saline to avoid contamination by platelets before to be lysed in 400 µL of RIPA buffer (Hepes 50 mM, NaCl 150 mM, EDTA 5 mM, NP40 1%, a cocktail of protease inhibitors 1%). After centrifugation, levels of proteins and BDNF (expressed as ng/mg of protein) in supernatants were determined using the Lowry method and an ELISA kit (Biosensis, BEK-2211-2P) according to manufacturer's instructions, respectively. Serum BDNF levels (expressed as ng/mL) were measured with the same ELISA kit.

Study outcome

Modified Rankin Scale (mRS) score at 3 months after hospital admission were collected via telephone interview. A favorable clinical outcome was defined as a mRS score of 0 to 2 and an unfavorable outcome including death as a mRS score of 3 to 6.

Statistical analysis

The results were expressed as percentages for categorical variables and as medians [interquartile ranges (IQR)] for continuous variables. Comparisons of categorical variables were performed using a $\chi 2$ test to which the Yates correction while comparisons of continuous variables were performed using a non-parametric Mann–Whitney *U* test. Differences between D0, D1 and D3 were assessed using repeated measures ANOVA test followed by Bonferroni's correction. The influence of BDNF levels in PBMC on NIHSS and mRS was performed by univariate and multivariate logistic regression analysis. Results were expressed as adjusted odds ratios (OR) with the corresponding 95 % confidence interval (95%

CI). Receiver operating characteristic analysis was used to determine the optimal BDNF cutoff that maximized the sum of sensitivity and specificity and results were reported as area under the curve (AUC). All statistical analyses were performed using Sigmaplot®11.0 software. Statistical significance was defined as $p < 0.05$.

Results

Patient's characteristics

Patient's characteristics are summarized in Table I. In a total of 40 patients, 62.5 % had a favorable outcome with a median mRS of 1. In other patients including dead patients (n=3 between hospital discharge and month 3), the median mRS reached 3. Statistical analysis showed that demographics and stroke etiology did not differ among patients with favorable and unfavorable outcome. By contrast, ESC score, the delay between admission and fibrinolysis and stroke severity from hospital admission to discharge was significantly lower in patients with favorable outcome than in patients with unfavorable outcome. Concerning hematological parameters, patients with favorable outcome exhibited lower lymphocytes count and higher neutrophils/lymphocytes ratio as compared to patients with unfavorable outcome. Importantly, platelet count did not differ between the groups.

Association between BDNF either in serum or in PBMC and stroke outcome

Consistent with a lack of difference in platelet count between patients with favorable and unfavorable outcome, BDNF levels in serum did not differ between the 2 groups of patients from admission to D3 (Table I). By contrast, BDNF levels in PBMC were significantly higher in patients with favorable than in patients with unfavorable outcome at least at day 3 after admission. Indeed, at earlier times (D0 and D1), no difference was observed between groups (Table I). Importantly, while BDNF levels in PBMC decreased from D0 to D3 in patients with unfavorable outcome (- 64%, p=0.013), the levels did not show significant changes (-25%,

p=NS) in patients with favorable outcome. We then more precisely explored the relationship between BDNF levels in PBMC at D3 and stroke outcome. Among patients with PBMC-BDNF levels within the third tertile, 92% had a favorable outcome while this percentage dropped to 53.4% and 46.1% when BDNF levels in PBMC were within the first and second tertile, respectively (Fig.1A). Univariate logistic regression analysis showed that PBMC-BDNF levels were strongly associated with good outcome (OR=12.0; 95% CI=1.4-106.2; p=0.023). Multivariate models indicated that PBMC-BDNF levels remained a predictor of stroke outcome (OR=12.4; 95% CI=1.4-112.2; p=0.046) after adjusting for other predictors (ESC score, interval between admission and fibrinolysis, NIHSS from hospital admission to exit, lymphocytes and neutrophils/lymphocytes ratio at admission). Finally, based on ROC curve (Fig.1B) and AUC (0.703, 95% CI: 0.533-0.873; $p<0.05$), the optimal cutoff value of PBMC-BDNF levels to predict a good outcome was 6.66 ng/mg protein with a sensitivity and specificity at 48.0% and 92.9%, respectively.

Discussion

The present study shows that acute BDNF levels in PBMC of stroke patients are higher in patients with favorable than in patients with unfavorable outcome as assessed from mRS at month 3 after adjusting for variables that have been associated with mRS including cardiovascular health status, delay of fibrinolysis, NIHSS from hospital admission to discharge, lymphocytes count and neutrophils to lymphocytes ratio. It also confirms our previous study showing that mRS at month 3 is not influenced by serum BDNF levels, irrespective from time sampling (from admission to day 3 after admission).

The role of PBMC on the ischemic brain is complex and not well understood. They exert both adverse and beneficial effects, which totally depend on injury severity, time window and cell subsets [13, 14]. While cytokines secreted by infiltrated PBMC (IL6, TNFα) have been largely involved in their adverse effect, the mediators underlying their beneficial

effect in stroke remain largely speculative. The present study supports the idea that infiltration of the ischemic brain by PBMC might contribute to functional recovery and that this effect likely relates to their ability to produce and deliver BDNF to the ischemic brain. Consistently, BDNF levels in PBMC > 6.66 ng/mg of proteins at day 3 after hospital admission were associated with a 12.4- fold increase in favorable outcome. These data fit well with the association observed between impaired cognition and reduced BDNF production by PBMC in patients with multiple sclerosis [15]. They also resonate with a recent study that reported a better outcome at month 6 after stroke onset in patients with high percentages of CD4(+) BDNF(+) Treg cells 24 hours after stroke onset than in those with lower levels [16]. In the present study, BDNF levels in PBMC decreased from admission to D3 in patients with bad recovery, but remained stable in patients with good recovery, suggesting that recovery after stroke might be dependent on still unidentified circulating factors able to inhibit BDNF synthesis by PBMC.

Some limitations of the present study should be considered. First, all stroke patients enrolled in the present study benefited from fibrinolysis which is expected to restore blood and PBMC supply to the ischemic region. However, success of reperfusion was not controlled. Second, genetic variation in the BDNF gene was not tested in the present study, while the Val66Met polymorphism was associated with poor motor recovery in stroke patients [17]. However, BDNF secretion by immune cells was reported to be independent on the Val66Met polymorphism [18].

In conclusion, the present study suggests that PBMC may be envisaged as an attractive vector to supplement the ischemic brain with BDNF. A future challenge is to identify factors that inhibit and induce BDNF synthesis by PBMC in stroke.

Acknowledgments

Sources of Funding

Work was supported by funding from the University of Bourgogne - Franche Comté and INSERM.

Conflict(s)-of-Interest/Disclosure(s)

Disclosures: None

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Figure legends

Figure 1. A) Distribution of modified Rankin scale (mRS) scores at month 3 according to the tertiles of BDNF levels in peripheral blood mononuclear cells (PBMC) at day 3 after stroke onset. B) Receiver operating characteristics (ROC) curve was utilized to determine the optimal cutoff value of PBMC-BDNF levels to predict a good outcome.