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A large screening of angiogenesis biomarkers and their association with neurological outcome after ischemic stroke

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ABSTRACT

Background: The induction of angiogenesis after stroke may enhance neurorestorative processes. Our aim was to examine the endogenous angiogenesis balance and their association with long-term clinical outcome in ischemic stroke patients.
Methods: A total of 109 stroke subjects were included in the study. Firstly, plasma samples were obtained from control subjects ($n = 26$) and tPA-treated stroke patients ($n = 29$) at baseline (within 3 h of symptoms onset), 1, 2, 12, 24 h after tPA treatment, at discharge and 3 months after the ischemic event. Angiogenic promoters (PDGF-AA, PDGF-BB, HGF, FGF, KGF, HB-EGF, TPO, VEGF, VEGFR-1, VEGFR-2 and SDF-1α) and inhibitors (endostatin, angiostatin, thrombospondin-1 and thrombospondin-2) were analyzed by Searchlight® technology or ELISA. Additionally, baseline and 24 h endostatin plasma level was determined in a new set of stroke patients ($n = 80$). Clinical parameters (NIHSS, mRS, mortality and hemorrhagic transformation events) were assessed to evaluate outcome.
Results: Baseline PDGF-BB, endostatin and thrombospondin-2 levels were higher in stroke patients than in controls ($p < 0.05$). A pro-angiogenic balance was associated with lower NIHSS scores and less intracranial hemorrhagic complications. Interestingly, a high baseline endostatin level was associated to long-term functional dependency (mRS > 2; $p = 0.004$). Finally, a baseline endostatin cut-off point of 184 ng/mL was an independent predictor of functional dependency at three months in the multiple logistic regression with an odds ratio of 8.9 (95% CI: 2.7–28.8; $p = 0.0002$).
Conclusions: Our results indicate that an early pro-angiogenic balance is associated with mild short-term neurological deficit, while an acute anti-angiogenesis status determined by high endostatin plasma level predicts a worse long-term functional outcome.

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1. Introduction

Stroke is the third leading cause of death and the most common cause of permanent disability in adults worldwide. Currently, the only available treatment for acute ischemic stroke is the intravenous administration of tissue plasminogen activator (tPA) up to 4.5 h after symptoms onset. However, despite its proven efficacy, tPA is administered to less than 5% of stroke patients due to its narrow therapeutic time-window and its increased risk of intracranial hemorrhage [1]. These data suggest that there is an urgent need for new stroke therapies applicable beyond the hyperacute phase. In this scenario, cerebral neovascularization after ischemia could be one promising strategy [2,3].

The use of blood biomarkers is becoming increasingly accepted in cerebrovascular diseases since biomarkers might aid physicians in several steps of stroke evaluation. Injury-related pathological pathways such as inflammation, reperfusion injury, hemorrhagic events and others have been the main focus to search for potential biomarkers [4,5]. Restorative therapies based on neuro-angiogenic phenomena that endogenously occur after the ischemic event might also provide useful biomarkers [6,7]. New blood vessels in the adult can be formed through the different processes of angiogenesis, arteriogenesis and vasculogenesis and might thus participate in remodelling the damaged area during the subacute phase [8]. Previous studies have demonstrated that FGF and VEGF remained increased during the first two weeks after stroke [9,10]. Biomarkers that could be measured during the delayed phase, to avoid acute phase changes, could be a promising ther-

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apeutic approach from which would benefit a larger number of patients.

A better understanding of the temporal profile of pro- and anti-angiogenic factors in stroke patients can add relevant information to the field. Our hypothesis was that the temporal modulation of angiogenesis could be a promising therapeutic approach to obtain maximal neuroreparative benefits following ischemic stroke. Thus, the aim of our study was to examine plasma levels of a large number of promoters and inhibitors of angiogenesis and their association with long-term clinical outcome in tPA-treated stroke patients.

2. Materials and methods

2.1. Study population

The prospective study included stroke patients ($n = 109$) through two steps: a first phase was an angiogenesis screening to establish a temporal profile ($n = 29$) and the second phase included more patients ($n = 80$) to evaluate baseline and 24 h endostatin level. All patients had an acute ischemic stroke involving the middle cerebral artery (MCA) territory admitted to the emergency department of a teaching hospital. All patients received thrombolytic therapy in a standard dose of 0.9 mg/kg within 3 h of symptoms onset. On admission, all patients underwent a cranial computed tomography (CT) scan and repeated 24–48 h later (or earlier when rapid neurological deterioration occurred) to evaluate the presence and type of hemorrhagic transformation (HT), defined according to previously published criteria [11]. A detailed history of vascular risk factors, drug abuse, and concomitant medication was obtained from each patient. National Institute of Health Stroke Scale (NIHSS) score was recorded to assess neurological status at admission and during follow-up visits [1, 2, 12, 24, 48 and at discharge (between 3 and 7 days)]. Neurological improvement was defined as a decrease in NIHSS $\leq$4 points and neurological deterioration as either death or increase in NIHSS $\geq$4 points at 48 h [12]. Functional outcome was defined by modified Rankin Scale (mRS) at three months [13]. Patients were considered dependent when mRS was >2 points. Transcranial Doppler (TCD) examinations were performed before the beginning of the treatment, at the end of tPA administration and serially for the first 24 h. Proximal or distal MCA occlusions and follow-up recanalization degrees were defined as previously described [14]. Additionally, an age- and sex-matched control group ($n = 26$) of subjects not suffering from any inflammatory or infectious disease was studied to obtain a control range of the studied promoters and inhibitors. The study was approved by the local Ethics Committee and conducted in accordance with the Declaration of Helsinki. All participants or relatives gave informed, written consent.

2.2. Immunoassay methods

Blood samples were drawn from each patient (or control) at admission (baseline) before any treatment was initiated. A temporal profile was determined at different time points: 1 h after treatment, 2 h, 12 h, 24 h after symptoms onset, at discharge (between 3 and 7 days) and at three months. Plasma was extracted in EDTA tubes, immediately separated by centrifugation at 3500 rpm for 15 min and stored at −80 °C until analysis.

Quantitative measurement of human angiogenic factors was performed using different protein arrays (Searchlight®, Aushon Biosystems, MA, USA) to detect plasma level of platelet derived growth factor BB (PDGF-BB), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), heparin binding epidermal growth factor like growth factor (HB-EGF), keratinocyte growth factor (KGF), platelet derived growth factor AA (PDGF-AA), thrombopoietin (TPO), vascular endothelial growth factor receptor 1 (VEGFR-1), vascular endothelial growth factor receptor 2 (VEGFR-2), thrombospondin 1 (TSP-1) and thrombospondin 2 (TSP-2). The level of SDF-1α, endostatin (Quantikine, R& D Systems, MN, USA) and angiostatin (Raybiotech, GA, USA) was determined by enzyme-linked immunosorbent assay (ELISA). Each sample was analyzed twice and the mean of the two values was used. The mean intra-assay coefficient of variation (CV) was lower than 20% for all biomarkers measured.

2.3. Statistical analysis

Angiogenic promoters and inhibitors did not follow a normal distribution (Kolmogorov-Smirnov and P-P plot). Angiogenic balance was assessed with the ratio between the level of promoter and inhibitor analyzed. Statistical significance for intergroup differences was assessed by Kruskall–Wallis and Mann–Whitney test. To study correlations between continuous variables, Spearman coefficients were used. A repeated measurement test (Wilcoxon) was used to analyze significant increases/decreases on the temporal profile of the studied molecules. Logistic regression was performed to determine factors that could be considered as independent predictors of dependency at three months (mRS > 2), using the forward step-wise method by the likelihood ratio test. The odd ratios (ORs) and 95% confidence intervals (CIs) for the effect on dependency at three months were estimated using logistic regression analysis adjusted for the effects of conventional risk factors. All statistical analysis was conducted using SPSS© 15.0 (SPSS Inc. Chicago, IL, USA). To account for multiple statistical testing in temporal profiles, Bonferroni's correction was applied. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Population characteristics

Demographic characteristics and risk factors profile of our studied populations are described in Table 1. Mean age of stroke patients was 70.9 years and 55% were male. Concerning risk factors, 24.1% of stroke patients were smokers, 10.4% had diabetes mellitus and 3.4% of patients had a coronary artery disease. Dyslipidemia was significantly higher in controls (42.3%) than in patients (17.2%, $p = 0.045$). However, in the larger population, dyslipemia did not differ between controls and patients ($p = 0.152$). Hypertension was significantly higher in stroke population ($n = 109$; $p = 0.039$) compared to controls. Etiology of stroke defined by the TOAST criteria revealed that about 60% of cases were of cardioembolic etiology, 20% were atherothrombotic and 20% were of undetermined cause.

3.2. Endogenous angiogenic promoters and inhibitors after ischemic stroke

The plasma temporal profiles of nine angiogenic promoters are shown in Fig. 1A. Baseline PDGF-BB level in strokes was 250.1 (171.9–588.1) pg/mL and the interval observed in healthy controls was 93 (49.1–203) pg/mL, $p = 0.003$. Overall, the growth factors PDGF-BB, PDGF-AA and VEGF all showed a similar profile, with a decrease during the 2–12 h period and an increase after 24 h. The angiogenic promoter SDF-1α presented a peak during the first hour post-thrombolytic treatment and gradually decreased during the following 7 days. TPO and HB-EGF plasma level in both stroke and control subjects could not be detected because plasma levels were under the detection limit of the technique (11.72 pg/mL for TPO and 3.66 pg/mL for HB-EGF).

Fig. 1B shows the results obtained for the four angiogenic inhibitors. Baseline endostatin level was significantly higher in stroke patients than in controls [161.6 (126.7–198.9) ng/mL vs. 141

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