Journal of Neuroimmunology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Journal of Neuroimmunology

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

Tim-4 expression increases in ischemic stroke patients and is associated with poor outcome

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ARTICLE INFO

Keywords:
Ischemic stroke
T cell immunoglobulin and mucin domain 4
Stroke associated infection
Monocytes

ABSTRACT

T cell immunoglobin and mucin domain (Tim)-4 on monocytes is involved in immune regulation. Here, we investigated Tim-4 expression on circulating monocytes and in plasma of ischemic stroke. Tim-4 expression was significantly increased on day 2 and day 5 after stroke. Furthermore, stroke severity was positively correlated with Tim-4 expression on monocytes or in plasma. Increased Tim-4 expression was related to stroke associated with infection (SAI) on day 2. Up-regulated Tim-4 expression on monocytes or in plasma on day 2 was a risk predictor of outcome. Our findings suggest that Tim-4 can act as a prognostic biomarker of ischemic stroke.

1. Introduction

Stroke is a common disease and is the second leading cause of death worldwide. Ischemic stroke, accounting for 60%–80%, is the most common type of stroke. It has a high disability rate and high mortality, which has caused a serious burden on family and society. There is plenty of evidence that immunity and inflammation participate in the pathogenesis of ischemic stroke (Urra et al., 2009a,b; Iadecola and Anrather, 2011; Kaito et al., 2013). The infiltration of inflammatory cells, such as macrophage, has been observed in the models of ischemic stroke (Foulkes et al., 1988). Additionally, the number of peripheral monocytes and proinflammatory cytokines (tumor necrosis factor-α, interleukin-6 etc.) are also significantly increased in ischemic stroke patients (Urra et al., 2009a,b; Cui et al., 2012; Pusch et al., 2015).

Therefore, a better understanding of potential molecular mechanisms of altered monocytes activity in ischemic stroke is necessary.

Tim-4 encodes a 60KD typeItrans-membrane protein (Shakhov et al., 2004) and also has a secretory form (Feng et al., 2008). Unlike other Tim molecules, Tim-4 was mainly expressed on antigen-presenting cells (APCs), especially highly expressed on macrophages and dentritic cells (DC) but not in T cells (Rodriguez-Manzanet et al., 2008), indicating that Tim-4 play important roles on these cells. Tim-4, a counter-ligand, was initially found to promote the proliferation of T cells through binding with its ligand Tim-1 (Meyers et al., 2005; Umetsu et al., 2005). Recent studies have shown that Tim-4 was involved in the regulation of macrophage activity. Tim-4, as a phosphatidylserine (PS) receptor, could enhance phagocytosing activity of apoptotic cells by macrophages (Miyanishi et al., 2007; Kobayashi et al., 2007). *In vitro*, macrophage can generate proinflammatory cytokines in response to Tim-4-Ig protein (Hein and Woods, 2007). Abe et al. (2013) revealed that anti-Tim-4 antibody–treated mice with arthritis exhibited a concomitant decrease in proinflammatory cytokines in the joints. These researches suggest that Tim-4 dysregulation is associated with excessive or uncontrolled inflammation and diseases. In this study, we will explore the relationship between Tim-4 expression and ischemic stroke, which was closely related to monocyte/macrophage-mediated inflammatory response.

To explore the association between Tim-4 expression and ischemic stroke, we compared Tim-4 expression on monocytes and in plasma between stroke patients and control group, and investigated the time courses of Tim-4 expression in patients. Moreover, the relationships between Tim-4 expression and outcome of patients were also evaluated. Our results showed that Tim-4 expression increased in ischemic stroke patients and was associated with poor outcome.

2. Materials and methods

2.1. Patients

Ischemic stroke patients, who were hospitalized within 24 h after stroke from March 2014 to June 2015 in Zhoushan hospital, were recruited into this study. All patients were < 80 years old, had a modified

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https://doi.org/10.1016/j.jneuroim.2017.11.017
Received 13 October 2017; Received in revised form 29 November 2017; Accepted 29 November 2017

Table 1
Baseline characteristics of stroke patients and control group.

	Stroke patients (N = 68)	Control group (N = 57)	P
Age, median (interquartiles)	69.1 (59.0–76.5)	68.7 (60.9–76)	0.98
Male, n (%)	39 (57.4%)	30 (52.6%)	0.28
Current smokers, n (%)	36 (52.9%)	25 (43.9%)	0.31
Hypertension, n (%)	53 (77.9%)	21 (36.8%)	< 0.001
Diabetes mellitus, n (%)	15 (22.1%)	7 (12.3%)	0.15
Dyslipidemia, n %	51 (75.0%)	30 (52.7%)	< 0.01
Ischemic heart disease, n (%)	11 (16.2%)	2 (3.5%)	0.04
Atrial fibliration, n (%)	19 (27.9%)	1 (1.8%)	< 0.001
Prior stroke, n (%)	8 (11.0%)	–	–
Chronic heart failure, n (%)	17 (25.0%)	–	–
Chronic kidney disease, n (%)	5 (7%)	–	–
NIHSS score on admission, median (interquartiles)	11.0 5.0–17.0	–	–
CRP (mg/L)	13.5 (4–45.2)	4 (0–8)	< 0.01
TOAST subtype			
Large-vessel disease	24 (35.3%)	–	–
Cardioembolic	12 (17.6%)	–	–
Small-artery disease	13 (19.1%)	–	–
Other known	10 (14.7%)	–	–
Undetermined	9 (13.2%)	–	–
SAI, n (%)	23 (33.8%)	–	–
Pneumonia, n (%)	12 (17.6%)	–	–
Urinary tract infection, n (%)	11 (16.2%)	–	–
PO, n (%)	40 (58.8%)	–	–

NIHSS: National Institute of Health Stroke Scale; SAI: Stroke Associated Infection; PO: Poor Outcome.

Rankin Scale score ≤ 2 prior to stroke. Patients in our study didn't receive thrombolytic therapy because the time from symptom initiation to admission was > 4.5 h. Only 6 patients used platelet aggregation inhibitors (aspirin) from symptom initiation to admission, and 7 patients had standardized use of platelet aggregation inhibitors (aspirin) and 61 patients rarely used platelet aggregation inhibitors as primary prevention. All the patients with stroke were treated with platelet aggregation inhibitors (aspirin, clopidogrel) as secondary prevention. Exclusion criteria included concurrent infection upon admission, the use of antibiotics, steroids, or immunosuppressants within the preceding three months, and recent (three months) history of chronic inflammatory diseases, tumor bearing state, immunological diseases, encephalopyosis, or raniocerebral trauma. From 116 consecutive ischemic stroke patients, 97 patients fulfilled inclusion criteria. We excluded 29 patients who did not give consent for participation in the study. Finally, there were 68 patients included in our study. The baseline characteristics of stroke patients and control group are shown in Table 1. SAI including pneumonia (N = 12) and urinary tract infection (N = 11), which occurred in 23 stroke patients at a mean of 2.2 days after admission. In addition, there were 40 stroke patients with poor outcome (PO), which included 23 SAI patients. Control group consisted of 57 age-matched non-stroke subjects who were admitted to our hospital for physical examination and didn't suffer with other inflammatory diseases, cancer, autoimmune disease, and a serious liver, renal, haematological or neurological disorder. This study was approved by the ethics committee of Zhoushan Hospital, China. All participants or legal guardians provided a signed written informed consent. Ischemic stroke was confirmed by computed tomography (CT) or magnetic resonance imaging (MRI). Neurological impairment was evaluated with National Institute of Health stroke scale (NIHSS) scores from admission to day 7, and at day 90, and functional outcome was assessed at day 90. Unfavorable outcome was defined as mRS 3–6 and favorable outcome as mRS 0–2. According to The Early Systemic Prophylaxis of Infection After Stroke (ESPIAS) study, stroke-associated infection (SAI) was defined as having a body temperature > 37.8 °C in patients with suggestive symptoms (*i.e.* cough, dyspnea, pleuritic pain and urinary tract symptoms), or white blood cell count > 11.0×10^9/L or < 4.0×10^9/L, pulmonary infiltrate on chest X-rays, or culture positive for a pathogen during the first week of stroke.

2.2. Neuroimaging

CT scan was performed with a 64-detector row CT scanner (Toshiba, Japan). MRI was conducted with a 1.5 T scanner (Siemens, Japan).

2.3. Flow cytometry

Blood samples were immediately collected from enrolled ischemic stroke patients upon admission before any medication was taken (0 day), and between 06:30 and 07:30 at the 2nd, 5th and 7th day of admission in the hospital. Finally, stroke patients were followed up at day 90 after stroke. In addition, blood samples of control group were collected. Flow cytometry was performed to detect Tim-4 expression on monocytes. The following monoclonal antibodies were used: PE-conjugated mouse anti-human Tim-4 (Clone Number: 9F4; BioLegend, San Diego, USA), PE-conjugated mouse IgG1, κ isotype ctrl (Clone Number: MOPC-21; BioLegend, San Diego, USA), FITC-conjugated mouse anti-human CD14 (Clone Number: M5E5; BD Biosciences, San Jose, USA). 5 μl specific monoclonal antibody or isotype control was mixed with 100 μl of whole blood, and incubated for 30 min at 4 °C in a dark room. After staining, blood specimens were processed for erythrocyte lysis using 1:10 FACS lysis solution (BD Biosciences, San Jose, USA). Cells labeled with monoclonal antibodies were collected and washed twice with phosphate buffer solution (PBS), performed on a BD FACSCalibur™ flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR, USA).

2.4. ELISA

Peripheral blood samples were centrifuged for 10 min at 1000 × *g*, and plasma specimens were collected and stored at − 80 °C for later use. Plasma Tim-4 concentration was detected using enzyme linked immunosorbent assay (ELISA) kit (Catalog number: CSB-EL023546HU, Cusabio, Wuhan, China) according to manufacturer's instructions. According to standard curves of the correlation between optical density and molecular concentration, calculated results were expressed as nanogram per milliliter. C-reactive protein (CRP) level was obtained by immunoturbidimetry (Requirements for Immunological turbidity kit for human C-reactive protein, BECKMAN COULTER IMMAGE 800, USA). The assays were performed according to the manufacturer's instructions.

2.5. Statistical analysis

All data were analyzed using SPSS software version 21.0 (SPSS Inc.). Student's *t*-test, Fisher exact test, Mann–Whitney *U* test, and analysis of variance (ANOVA), were used for comparison. Correlations were calculated with Spearman Rank correlation coefficient. $P < 0.05$ was considered statistically significant.

3. Results

We first detected Tim-4 expression on monocytes and compared the difference between stroke patients and controls. Representative cases of flow cytometry are shown in Fig. 1A and B. Our results showed that Tim-4 expression on monocytes of stroke patients significantly increased on day 2 and day 5 (on day 2, stroke patients *vs* control, 42.5% ± 9.35% *vs* 23.07% ± 8.06%, $P < 0.01$; on day 5, stroke patients *vs* control, 37.31% ± 9.56% *vs* 23.07% ± 8.06%, $P < 0.01$)