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Plenary article

Pro-inflammatory cytokine network in peripheral inflammation response to cerebral ischemia

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HIGHLIGHTS

- The pro-inflammatory cytokines network after stroke is poorly understood.
- 8 plasma cytokines and mRNA levels were measured within 72 h after stroke.
- Cytokine interactions were analyzed using the Bayesian network.
- Only the elevation of IL-6 correlated with the severity and prognosis of stroke.
- IL-6 appears to be the key mediator of pro-inflammatory cytokines network.

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ABSTRACT

The key circulating pro-inflammatory cytokines and their interaction in peripheral inflammation after acute cerebral ischemia are poorly understood. CD40L, IFN-γ, IL-1α, IL-1β, IL-6, IL-8, IL-17 and TNF-α were determined using multi-ELISA kit in stroke patients within 72 h of an acute ischemic attack. Leukocyte mRNAs were determined using real-time polymerase chain reactions (PCR). Stroke severity and clinical outcomes were evaluated by National Institutes of Health Stroke Scores (NIHSS) and modified Rankin Scale (mRS). Plasma/mRNA cytokine interactions were analyzed using the Bayesian network learning procedure. Compared to controls, stroke patients had higher IL-6, IL-8 and TNFα protein in plasma and lower IL-6, IL-8, TNFα, IL-1α, and IL-1β mRNA in leukocyte within 72 h after stroke. However, only the elevation of IL-6 correlated with the severity and prognosis of their stroke. This was associated with a decreased IL-6 mRNA in leukocyte. Further study showed that Bayesian network analysis revealed that changes in the other cytokines were subsequent to IL-6 leukocyte cytokine RNA. The change of other cytokines in plasma proteins after ischemic brain injury appeared secondary to IL-6. Pro-inflammatory cytokines up-regulation in plasma and compensatory immunity depression in leukocyte involve in peripheral inflammation response to cerebral ischemia. IL-6 appears to be the key mediator of circulating pro-inflammatory cytokines network.

1. Introduction

Stroke is a leading cause of death and disability worldwide. Numerous studies have demonstrated that the peripheral and central inflammatory response plays a crucial role in brain damage following ischemic injury [2,10]. Circulating pro-inflammatory cytokines in stroke patients are markedly altered in response to the brain injury [1,11,18,20]. To date, the most studied pro-inflammatory cytokines include IL-1β, TNFα [5], IL-6 [17,21], IL-8 [9], CD40L [3], IFNγ, IL-1α and IL-17 [16]. However, the interaction of these circulating pro-inflammatory cytokines in stroke patients is poorly understood. The identity and source of the most important cytokine remains elusive. This is important for possible anti-inflammatory therapy of stroke patients in the future. In the present study, the relationships between the clinical features of cerebral ischemia and the plasma levels of the cytokines and their mRNA levels in leucocytes were determined. We further explored and analyzed the characteristics of circulating cytokine interactions in order to understand their roles in the pathophysiological process.

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2. Materials and methods

2.1. Study subjects

Venous blood sampling involved for this study was approved by the Institutional Review Board of Shanghai Jiao Tong University, Shanghai, China. Each patient or volunteer gave informed consent. Patients with first-ever acute ischemic strokes within 72 h from the onset of symptoms were recruited from the Department of Neurology, Ruijin Hospital School of Medicine, Shanghai Jiao Tong University, Shanghai, China. Acute ischemic stroke was diagnosed by two neurologists based upon the patient history, neurological deficit, and MRI imaging. Patients with acute infectious diseases, immunological diseases, blood disorders, severe renal or liver failure, tumor history, and recurrent stroke were excluded. Stroke severity was evaluated using the National Institutes of Health Stroke Severity (NIHSS) scale on the day of blood sample collection. The patients were treated according the guideline for the management of ischemic stroke in China. Clinical outcomes were obtained using a modified Rankin Scale (mRS) three months later. Control blood samples were obtained randomly from normal stroke-free volunteers. Their age, gender, and incidence of cardiovascular risk factors were matched with the stroke patient group.

2.2. Blood sample collection

Blood samples (4 ml) from each patient were collected and placed into EDTA containing tubes (BD Vacutainer, Plymouth, UK) by venipuncture after their admission but before treatment. Blood samples were spun in a centrifuge at $3000 \times g$ for 15 min at room temperature. A plasma layer aliquot was obtained and stored at $-80\,^{\circ}C$. Red blood cells were lysed by red blood cell lysis buffer and discarded. The white cell buffy coat layer was collected for further analysis.

2.3. Measurement of plasma pro-inflammatory cytokines

Pro-inflammatory cytokines were determined by ELISA assay using the Mosaic™ ELISA human cytokine panel 1 kit (R&D Systems, Minneapolis, MN). Duplicate readings for each standard and sample were determined. The image acquisitions were taken by the *Bio-Rad* ChemiDoc XRS camera (Bio-Rad, Hercules, CA) and further analyzed by Q-View™ software (Quansys Biosciences, Logan, UT).

2.4. RNA extraction, reverse transcriptions (RT) and real time quantitative PCR

Total leukocyte RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA). The integrity and concentration of total RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific, UT). Samples with OD 260/280 ratio in the range of 1.7–2.0 were used. Reverse transcriptions of total RNA to cDNA were performed via a PrimeScript® RT reagent kit with gDNA Eraser (Perfect Real Time, Takara, Osaka). Real time quantitative PCR was performed by the fast real-time PCR system (7900HT, Applied Biosystems, ABI) using a SYBR® Prime ScriptTM RT-PCR Kit II (Perfect Real Time, Takara, Osaka). Primer sequences of the pro-inflammatory cytokines and endogenous control (β-actin) were obtained from the PrimerBank (Supplementary Table 1). Quantification of each cytokine was normalized to β-actin expression for each sample in triplicate.

2.5. Interactions among cytokines via the Bayesian network learning approach

Potential causal relationships among the pro-inflammatory cytokine proteins and their mRNA counterparts in the ischemic stroke patients were explored using the Bayesian network (BN) learning approach. This was used to construct a directed acyclic graph (DAG) for possible causal relationships among CD40L, IFN-γ, IL-1α, IL-1β, IL-6, IL-8, IL-17 and TNF-α protein in protein BN or their mRNA counterparts. A node in the DAG represented a corresponding protein or an mRNA counterpart. The BN connectivity models were in a complete data-driven search-and-score manner. A Bayesian information criterion (BIC) was employed to identify the BN model, optimizing the trade-off between likelihood and model complexity over the extent of all possible candidate BN configurations [14]. L1-Regularization Paths algorithms and maximum likelihood estimations were used for studying the structure and parameters of the BN model [13]. The details were implemented in MATLAB functions L1DAG Learn (http://www.cs.ubc.ca/~murphyk/Software/DAGlearn/) and Bayesian Net Toolbox (BNT, www.cs.ubc.ca/~murphyk/Software/BNT/bnt.html).

2.6. Statistical analysis

The data were first examined for a normal distribution using the Kolmogorov–Smirnov test. For non-normal distributing data, the comparison of two independent groups was analyzed by the non-parametric Mann–Whitney *U* test. Spearman correlations between two variables were performed. All data are presented as median (lower quartile; upper quartile). Conversely, normal distributing data was analyzed using the Student's *t*-test and Pearson's correlations. Data were presented as means ± standard deviations. Statistical significance was considered as $p < 0.05$. Sample size computation was performed during the study design [19].

3. Results

3.1. Clinical characteristics

A total of 115 stroke patients within 72 h of their acute ischemic attack, and 105 healthy controls were recruited (Supplementary Table 2). There were no significant differences in the median age and race/ethnicity between the two groups ($p > 0.05$). The incidence of risk factors was also matched between stroke patients and control groups including hypertension, diabetes, hyperlipidemia and cardiac diseases ($p > 0.05$). Among stroke patients, 49 patients were classified as large artery atherosclerotic stroke (LAAS), 20 as cardio-embolic infarct (CEI) and 46 as lacunar stroke according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification. Based on the Oxford Community Stroke Project (OCSP) classification via MRI imaging, strokes were classified as total anterior circulation infarct (TACI) 2, partial anterior circulation infarct (PACI) 42, posterior circulation infarct (POCI) 16, lacunar infarct (LACI) 55. In view of their mRS scores, the stroke patients were divided into a good outcome ($mRS \leq 2$, $n = 48$), and a poor outcome group ($mRS > 2$, $n = 66$, one withdrawn).

3.2. IL-6 is the unique circulating pro-inflammatory cytokine related to stroke severity and clinical outcome

The plasma cytokine levels were measured within the first 3 days after the patient's stroke. The IL-6, IL-8 and TNFα levels were increased significantly in the stroke patients compared to the healthy controls ($p = 0.004$, 7.09E−5, and 3.13E−5, respectively). In contrast, IFNγ, IL-1β and IL-17 plasma levels decreased after brain ischemia ($p = 0.048$, 2.09E−20, and 7.35E−7, respectively). No changes were found in CD40L and IL-1α levels (Fig. 1, $p = 0.103$ and 0.115, respectively). Among the pro-inflammatory cytokines, only IL-6 had a positive correlation with the NIHSS score in the patient group. The remaining cytokines including CD40L, IFNγ, IL-1α, IL-1β, IL-8, IL-17 and TNFα showed no correlation with the