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Cytokines in cerebrospinal fluid of neurosyphilis patients: Identification of Urokinase plasminogen activator using antibody microarrays

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ABSTRACT

Little is known regarding protein responses to syphilis infection in cerebrospinal fluid (CSF) of patients presenting with neurosyphilis. Protein and antibody arrays offer a new opportunity to gain insights into global protein expression profiles in these patients. Here we obtained CSF samples from 46 syphilis patients, 25 of which diagnosed as having central nervous system involvement based on clinical and laboratory findings. The CSF samples were then analyzed using a RayBioH L-Series 507 Antibody Array system designed to simultaneously analyze 507 specific cytokines. The results indicated that 41 molecules showed higher levels in patients with neurosyphilis in comparison with patients without neural involvement. For validation by single target ELISA, we selected five of them (MIP-1a, I-TAC/CXCL11, Urokinase plasminogen activator [uPA], and Oncostatin M) because they have previously been found to be involved in central nervous system (CNS) disorders. The ELISA tests confirmed that uPA levels were significantly higher in the CSF of neurosyphilis patients (109.1 ± 7.88 pg/ml) versus patients without CNS involvement (63.86 ± 4.53 pg/ml, $p < 0.0001$). There was also a clear correlation between CSF uPA levels and CSF protein levels ($p = 0.0128$) as well as CSF-VDRL titers ($p = 0.0074$) used to diagnose neurosyphilis. No significant difference between the two groups of patients, however, was found in uPA levels in the serum, suggesting specific activation of the inflammatory system in the CNS but not the periphery in neurosyphilis patients. We conclude that measurements of uPA levels in CSF may be an additional parameter for diagnosing neurosyphilis.

1. Introduction

Treponema pallidum (*T. pallidum*) is a bacterial spirochete that causes syphilis. This infectious agent is able to survive in the untreated human host for several decades, eventually causing damage to multiple organs (Salazar et al., 2002; Lafond and Lukehart, 2006). The central nervous system (CNS) is often involved as *T. pallidum* can spread to the CNS within days of exposure to the bacterium. This can subsequently result in a neurological disorder called neurosyphilis (Marra, 2009). The clinical and laboratory diagnosis of neurosyphilis is often difficult as the antigens associated with neurosyphilis infection are highly variable and the underlying pathogenesis is poorly understood. Currently, the diagnosis of neurosyphilis is confirmed by a positive cerebrospinal fluid (CSF) Venereal Disease Research Laboratory test (VDRL) (Workowski et al., 2010). This test, however, is only moderately sensitive, may be unavailable in resource-limited countries, and is cumbersome and time-consuming to perform. Consequently, in VDRL-negative patients, one has to rely on other laboratory findings for a presumptive diagnosis, such as the presence or absence of CSF pleocytosis or elevated protein concentrations.

Recently, the role of cytokines in the regulation of inflammation and host responses to CNS infection has attracted attention (Mukai et al., 2006; Griffiths et al., 2012). Cytokines are a group of important inflammatory mediators that act in cascades, inducing or inhibiting one another. In the CNS, cytokines are synthesized by microglia, astrocytes, and neurons indigenous to the brain. Cytokines are also synthesized by activated microglia that have migrated from the periphery as phagocytic cells (Banisadr et al., 2005). Many studies have attempted to define the cytokines whose expression levels in the CSF correlate with neurological disease progression, thus providing clues to the processes that lead to neuronal dysfunction. It has been suggested that IL-17, IL-17A and Interferon-γ are involved in central nervous system damage and inflammatory responses associated with neurosyphilis (Pastuszczak et al., 2013; Wang et al., 2014). Increased CSF levels of CXCL13 and CXCL12 may be particularly useful in the diagnosis of neurosyphilis in HIV-infected

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patients, due to the fact that these cytokines are independent of CSF pleocytosis and are markers of the HIV disease (Marra et al., 2010; Tsai et al., 2014). Nevertheless, as these studies focus on relatively few inflammatory cytokines, we still have an incomplete understanding of how interactions of *T. pallidum* with the CNS ultimately determine the extent of CNS dysfunction. To expand our knowledge about the nature of these interactions, we here used antibody-based arrays to examine the profile of 507 cytokines, chemokines, and growth factors in the CSF of neurosyphilis patients. Through statistical analysis and confirmation by ELISA, the levels of Urokinase plasminogen activator (uPA) were found to be significantly elevated in the CSF isolated from neurosyphilis patients when compared with syphilis patients without CNS involvement. Urokinase plasminogen activator is the active form of a serine protease that is derived from pro-uPA and that converts plasminogen into the active protease, plasmin. Plasmin subsequently breaks down components of extracellular matrix proteins (ECM) into active matrix metalloproteases (MMPs) either directly or through the activation of latent pro-matrix metalloproteases (pro-MMP). Extracellular proteolysis may indeed play a crucial role in the pathophysiology of neuronal cell death. Hence, the results of our study indicate that uPA may be involved in central nervous system damage associated with the clinical symptoms of neurosyphilis patients.

2. Materials and methods

2.1. Study participants

A retrospective review of patient records at Guangdong Provincial Dermatology Hospital was performed. Forty-six patients with newly diagnosed neurosyphilis or syphilis without CNS involvement were enrolled in the study between November 2012 and August 2014. Syphilis without CNS involvement was defined clinically by the absence of neurological or ophthalmological symptoms. This categorization also required positive serum treponemal serological test results [Toluidine red unheated serum test (TRUST), *T. pallidum* particle agglutination (TPPA)], and negative CSF-TRUST and CSF-TPPA test results. As previously published (Li et al., 2013), neurosyphilis was defined by reactive CSF-VDRL (Venereal Disease Research Laboratory) and CSF-TPPA tests in the absence of substantial blood contamination of the CSF as evidenced by the absence of erythrocytes upon microscopic examination at 400× magnification. Clinical neurological examinations included cranial nerve examination, muscle strength and muscle tension test, tendon reflexes, pathological reflex, and meningeal irritation sign determination.

Exclusion criteria were as follows: pregnancy, anti-inflammatory or immunosuppressive therapy, treatment with antibiotics within the last 6 months, and the occurrence of other chronic inflammatory disorders (eg. autoimmunity). All patients were HIV-negative and serologically positive for serum TRUST and TPPA.

The study protocol was reviewed and approved by the Ethics Committee at the Guangdong Provincial Dermatology Hospital. Written informed consent was obtained when collecting samples from all participants.

2.2. Laboratory investigations and CSF sampling

Lumbar puncture and CSF sampling was performed in all patients with syphilis. Cerebrospinal fluid samples were sent for routine examination [white blood cell (WBC) counts, glucose, and protein concentration] and serological testing (VDRL, TPPA and TRUST). The CSF-VDRL test was performed according to standard methods with modifications as described (Larsen et al., 1998). Aliquots of CSF were centrifuged and stored at −80 °C until cytokine measurements were performed. On the day CSF samples were collected, serum samples were also obtained from each patient and were tested for syphilis serology on the same day. Prior to cytokine measurements, serum aliquots were stored at −80 °C.

2.3. Human protein microarray assay

Prior to performing microarray analysis, samples were removed from the −80 °C freezer, thawed on ice, and briefly centrifuged. CSF from neurosyphilis patients (n = 3) was compared with CSF from syphilis patients where there was no CNS involvement (n = 3). The CSF samples were biotinylated and spotted onto RayBioHL-Series 507 Biotin Label-based Antibody Array chips (RayBiotech, GA, USA). In order to reduce batch variability, 100 μl of each undiluted CSF sample was assayed on a single chip. The array data were normalized based on the average positive control signal intensity of each array, and the median signal intensities of every spot were corrected for local background. The chip was read using a GenePix 4000B Microarray Scanner (Molecular Devices, Sunnyvale, CA, USA). The array was arranged so that each antibody was spotted twice, creating two technical replicates per protein of interest. Specific protocol details pertaining to the procedure can be found on the Ray Biotech Inc. website (http://www.raybiotech.com/l-series-507-label-based-human-array-1-glass-slide-2.html). Protein arrays were visualized using the Quansys Q-View imager and protein levels were determined using the Quansys Q-View imaging software.

2.4. Enzyme-linked immunosorbent assays

To validate data from the L Series 507 array analysis, selected proteins from the list of significantly modulated CSF protein markers detected in the array analysis were quantified using commercially available ELISA kits (for CXCL11 and uPA: R&D Systems, Minneapolis, MN; and for MIP-1α and OSM: Raybiotech, GA, USA) according to the manufacturer's instructions. A reference curve was generated for each assay using six serial dilutions of appropriate standards. The human blood serum preparations were diluted 1:3 using antibody dilution buffers. The CSF samples used were not diluted.

In order to measure the anti-uPA antibody, 100 μl of Tris-buffered saline (TBS) containing 0.5 mg/ml uPA (Novoprotein) was added to ELISA strips and incubated overnight at 4 °C. The assembled strips were washed four times with TBS buffer. The strips were blocked with TBS containing 0.2% bovine albumin for 2 h at 37 °C. They were then washed four times with TBS. A 1:3 dilution of serum (100 μl) or undiluted CSF (100 μl) was added to the strips and incubated for 2 h at 37 °C. After washing with TBS and incubating with 100 μl of TBS (containing 1 mg/ml horseradish peroxidase-conjugated monoclonal anti-human IgG) for 1 h at 37 °C, the strips were incubated for 30 min at room temperature with a substrate solution. The reaction was terminated by adding 50 μl of 50% H_2SO_4, and the optical density of the solutions was determined at 450 nm using a Uniskan II plate reader (MTX Lab Systems, USA). The relative concentrations of anti-uPA antibodies in the analyzed samples were expressed as the difference in the relative absorbance at 450 nm (average of three measurements) between the experimental samples and the blank control samples.

2.5. Statistical analysis

Statistical analysis was performed with the Statistica 7.1 PL package (StatSoft, Inc., 2005). Data are expressed as mean ± SD if not otherwise stated. Comparisons between groups were performed by the Student's *t*-test, and the associations between the individual parameters were measured using Pearson product–moment correlation coefficient. p values < 0.05 were considered statistically significant.

3. Results

3.1. Patient characteristics

The characteristics of the 46 patients are shown in Table 1. Twenty-six patients had laboratory-defined neurosyphilis and 21 patients were