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Abnormal gene and protein expression of inflammatory cytokines in the postmortem brain of schizophrenia patients

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ABSTRACT

Immune function abnormalities have been implicated in the pathophysiology of schizophrenia (SZ). This is primarily based on the observation that the levels of proinflammatory cytokines are significantly increased in the serum of SZ patients compared with normal control (NC) subjects. However, it is not known if similar cytokines abnormalities are also present in the brain of SZ patients. To further examine the involvement of inflammatory cytokines in the brain of SZ patients, we determined the protein and mRNA levels of TNF-α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-13, LTA and IL-1RA in the prefrontal cortex (PFC, Brodmann area 9) of SZ patients. We found that the protein and mRNA expression levels of the cytokines TNF-α and IL-6 are significantly increased and those of IL-10 are significantly decreased in the PFC of SZ patients. No difference in the protein and mRNA levels of IL-1β, IL-13, and IL-1RA was observed between SZ patients and NC subjects. The protein expression levels of IL-8 were significantly decreased and those of LTA were significantly increased in SZ patients, but no significant difference in the mRNA levels of IL-8 and LTA was observed between SZ patients and NC subjects. The levels of IL-2 were undetectable or very low in the postmortem brain of either SZ or NC subjects. These results suggest abnormalities of specific pro- and anti-inflammatory cytokines in the postmortem brain of SZ patients. These observations may have important implications in understanding the role of inflammatory cytokines in the pathophysiology of SZ.

1. Introduction

Immune function abnormalities have been implicated in the etiology and pathophysiology of schizophrenia (SZ). Several studies suggest that prenatal infections act as a risk factor for immune function alterations later in life, such as abnormal cytokine production and marked changes in cognitive and affective behaviors throughout the lifespan (Miller et al., 2013). Several other studies also reported increased microglial activation in SZ patients (Bayer et al., 1999; Schnieder and Dwork, 2011; Steiner et al., 2008).

Cytokines are major mediators of immune function and hence it is not surprising that cytokines have been widely studied in SZ. The suggestion that abnormalities of cytokines are associated with SZ is based on several lines of evidence. For example, the administration of cytokines, such as interferon (IFN)-γ, to rats and humans produces a syndrome known as 'sickness behavior' (Dantzer et al., 1999). The development of psychosis with IFN therapy have been generally observed and reported, as reviewed by Cheng et al. (2009) and by Crane et al. (2003). Also, the administration of cytokines to cancer patients is associated with side effects such as anxiety, depression, psychosis, mania and delirium (Capuron et al., 2001). That abnormalities of cytokines are associated with SZ is also supported by the findings that proinflammatory cytokines are abnormally expressed in the serum of SZ patients, as reviewed by Potvin et al. (2008) and by Zakharyan and Boyajyan (2014). The involvement of cytokines in SZ is also based on studies of behavioral effects after peripheral administration of cytokines (Cheng et al., 2009). However, it is not clearly understood if cytokine abnormalities are also present in the brain. Cytokines can either be synthesized in the brain or transported from the periphery (Dantzer et al., 2008). Therefore, it is quite possible that cytokine abnormalities may also be present in the brain and associated with the pathophysiology of SZ. The initial evidence that abnormalities of cytokines may be present in the brain is substantiated by the reports of abnormal levels of cytokines in the cerebrospinal fluid (CSF) of SZ patients (el-Mallakh et al., 1993; Garver et al., 1999; Garver et al., 2003; Soderlund et al., 2009).

Some studies suggest an abnormal expression of cytokines in the postmortem brain of patients with mood disorders and SZ (Fillman et al., 2013; Shelton et al., 2010; Volk et al., 2015). For example, Shelton et al. (2010) found abnormal expression of several proinflammatory cytokines in the postmortem brain (PFC) of depressed subjects. Dean et al.

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(2010) also reported abnormalities of soluble and membrane-bound tumor necrosis factor (TNF) in the postmortem brain of patients with major depressive disorders (MDD).

In light of this background, it was of interest to examine if alterations in inflammatory cytokines are present in the postmortem brain of SZ patients. Therefore, the aim of the present study was to examine mRNA and protein expression levels of inflammatory cytokines, such as TNF-α, interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10, IL-13, and lymphotoxin-A (LTA) in the PFC of SZ patients.

2. Methods

2.1. Subjects

The study was performed in the PFC (Brodmann area 9 [BA9] of 31 SZ patients (16 SZ suicide victims and 15 non-suicide SZ patients) and 24 NC subjects. Brain tissues were obtained from the Maryland Brain Collection at the Maryland Psychiatric Research Center, Baltimore, Maryland. Tissues were collected only after a family member gave informed consent. All tissues from NC and SZ subjects were grossly examined by experienced neuropathologists. Toxicology data were obtained by blood and urine samples analysis. All procedures were approved by the University of Maryland Institutional Review Board (IRB) and by the University of Illinois IRB.

2.2. Diagnostic method

Subject diagnosis was based on the Structured Clinical Interview for DSM-IV (SCID) (Spitzer et al., 1992). At least one family member and/or a friend, after giving written informed consent, underwent an interview. Diagnoses were made by a consensus of two psychiatrists from the data obtained in the interview, medical records from the case, and records obtained from the Medical Examiner's office. Normal control subjects were verified as free from mental illnesses using these consensus diagnostic procedures.

2.3. Determination of mRNA levels

2.3.1. RNA extraction

RNA was isolated from 100 mg of tissue using the TRIZOL (Invitrogen) reagent followed with DNase treatment, as per the manufacturer's instructions. RNA concentration and quality was determined using NanoDrop®ND-1000 (Thermo Scientific, Waltham, MA) and Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA) respectively. RNA integrity number (RIN) showed no significant difference between groups and was >7.0 for all samples.

2.3.2. Quantitative real-time PCR

Gene expression was determined using a two-step real-time RT-PCR (qRT-PCR) method, as previously described (Pandey et al., 2012). Briefly, 1 μg of total RNA was reverse transcribed using 200 units MMLV-reverse transcriptase in 50 ng random hexamers, 2 mM dNTP mix, 10 units ribonuclease inhibitor, with final reaction volume of 20 μl.

qPCR was performed using Pre-designed TaqMan gene expression assays (Applied Biosystems, Foster City, CA) for all target and housekeeping genes, on MX3005p sequence detection system (Agilent, Santa Clara, CA). The TaqMan assay IDs are listed in Table 1. To determine the stability and optimal number of housekeeping genes we used geNORM version 3.4 (PrimerDesign Ltd, UK) to test twelve commonly used reference genes of different functional classes in 10 samples from each test group (Vandesompele et al., 2002). geNorm analysis was used to determine number and stability of normalizing genes. β-Actin and GAPDH ranked as the most stable genes in our samples. PCR efficiency was tested over 5-log dilution series and confirmed that all target genes and housekeeping genes similar amplification efficiencies. For each primer/probe set, the PCR reaction is carried out using 10 μl of cDNA diluted 1:10 fold. Each qPCR plate includes a "no reverse transcriptase" and "no template" control to eliminate non-specific amplification. One sample, from each target gene is run on a gel to confirm specificity and all samples were run in triplicates. Target gene qPCR data is normalized to the geometric mean of β-actin and GAPDH and is expressed relative to the control samples using $2^{-(\Delta\Delta Ct)}$ method, where ΔΔCT = (CT target − CT internal control) Subject − (CT target − CT internal control) Control, and $CT_{\text{internal control}}$ is the geometric mean of ACTB and GAPDH CTs. Outliers were excluded if the normalized (delta C_t) values were >2 standard deviations from the group mean. Relative expression levels are reported as fold change and ΔC_t values are used for further statistical analysis (Applied Biosystems User Bulletin No. 2).

Table 1
TaqMan primers/probes used for qPCR analysis.

	TaqMan accession	Probe location (exon boundary)	Assay function
ACTB	Hs99999903_m1	1-1	Housekeeping (HK)
GAPDH	Hs99999905_m1	3-3	Housekeeping (HK)
TNF-α	Hs99999043_m1	1-2	Target gene
IL-1β	Hs01555410_m1	3-4	Target gene
IL-1RN (IL-1RA)	Hs00893626_m1	4-5	Target gene
IL-2	Hs00174114_m1	2-3	Target gene
IL-6	Hs00985639_m1	2-3	Target gene
IL8	Hs00174103_m1	1-2	Target gene
IL10	Hs00961622_m1	4-5	Target gene
IL13	Hs00174379_m1	1-2	Target gene
LTA	Hs99999086_m1	3-4	Target gene

2.4. Determination of brain protein levels using ELISA

Levels of proinflammatory cytokines (IL-1β, IL-6, TNF-α) were determined in brain homogenates (100 μl) by enzyme-linked immunosorbent assay (ELISA) using commercially available Quantikine® kits (R & D Systems, Inc., Minneapolis, MN) for human IL-1β, human IL-6, and human TNF-α, according to the manufacturer's instructions.

2.5. Determination of brain protein levels using western blot method

Immunolabeling of inflammatory cytokines (IL-8, IL-10, IL-13, IL-1RA, LTA) was determined by the Western blot method in membrane fractions, as described in detail in one of our earlier publication (Dwivedi and Pandey, 2000). Briefly, the brain samples of PFC were homogenized directly in Tris-HCl buffer (50 mM, pH 7.5). Equal volumes of membrane fractions isolated by this procedure (30 μg of protein in 20 μl) were separated from 4 to 12% Bis-Tris gel (Invitrogen, Grand Island, NY, USA) and transferred to nitrocellulose membranes (Amersham). The membrane was blocked for 1 h at room temperature with 5% non-fat milk in phosphate-buffered saline (pH 7.4) and the blots were initially developed using the polyclonal primary antibodies (1:1000 dilution) overnight and detected by using anti-rabbit IgG (Amersham) at (1:3000 dilution)for 4 h followed by the application of the ECL chemiluminescence western blotting kit (Amersham).

The membranes were stripped using stripping solution (Chemicon International, Temecula, CA) To normalize the data, β-actin level was measured in the same immunoblot using β-actin as the primary monoclonal antibody (1:5000 for 2 h) and anti-mouse IgG (1:5000 for 2 h) as the secondary antibody. The levels of inflammatory cytokine proteins were calculated as a ratio of the optical density of the primary antibody to the optical density of β-actin antibody. The following polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA): IL-8 (molecular weight 8 kDa), IL-10 (molecular weight 37 kDa), IL-13 (molecular weight 13 kDa), and IL-1RA (molecular weight 25 kDa). LTA polyclonal antibody (molecular weight 19 kDa) was purchased from, Abnova (Walnut, CA). Secondary antibodies were purchased from (GE Healthcare Life Sciences, Pittsburgh, PA).

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