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Proinflammatory cytokines and their membrane-bound receptors are altered in the lymphocytes of schizophrenia patients

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ABSTRACT

Abnormalities of protein levels of proinflammatory cytokines and their soluble receptors have been reported in the plasma/serum of schizophrenia (SZ) patients. To examine if SZ is also associated with the abnormal gene expression of cytokines and their membrane-bound receptors, we studied mRNA expression of proinflammatory cytokines and their receptors in lymphocytes of SZ patients and normal control (NC) subjects. We determined the protein and mRNA expression of proinflammatory cytokines and mRNA expression of their receptors in lymphocytes from 30 SZ patients and 30 drug-free NC subjects. The subjects were diagnosed according to DSM-IV criteria. Protein levels of cytokines were determined by ELISA, and mRNA levels in lymphocytes were determined by the qPCR method. We found that the mRNA levels of IL-6, TNF-α, IL-1R1, TNFR1, and TNFR2, but not IL-1β, IL-1R2, IL-1RA, IL-6R, or GP130 were significantly increased in lymphocytes of SZ patients compared with NC subjects. We also found that the protein expression of IL-6 and TNF-α, but not IL-1β, was also significantly increased in SZ patients compared with NC subjects. These studies suggest that in addition to the reported abnormalities of proinflammatory cytokines and their soluble receptors in the plasma of SZ patients, an abnormal gene expression of these cytokines and their membrane-bound receptors may be involved in the pathogenesis of SZ.

1. Introduction

It has been observed that the immune function, in general, and cytokine abnormalities in particular, are associated with the pathophysiology of schizophrenia (SZ) (Drexhage et al., 2010a; Hope et al., 2009; Zakharyan and Boyajyan, 2014). The abnormalities of the immune function in SZ are based on both direct and indirect evidence. For example, the administration of cytokines such as interferon (IFN) to rats causes a constellation of symptoms known as "sickness behavior" that includes cognitive changes, slowed cognitive speed, diminished social interactions, and reduced locomotor activity and executive functions (Dantzer et al., 1999). Also, the administration of cytokines, such as IFN to cancer patients induces symptoms known as sickness behavior (Capuron et al., 2001). Psychiatric side-effects associated with IFN therapy include anxiety, depression, psychosis, mania, and delirium (Cheng et al., 2009; Crane et al., 2003; Hosoda et al., 2000). The development of psychosis with IFN therapy has been reported and reviewed by Cheng et al. (2009).

That inflammatory processes are also involved in SZ (Dean, 2011) are based on the observation that proinflammatory cytokines, which are released from the immune cells as a result of inflammation or stress, are abnormal in the serum of patients with SZ (see reviews and meta-analyses by Modabbernia et al., 2013; Munkholm et al., 2013a,b; Potvin et al., 2008). There are several studies, although not always consistent, that report increased levels of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and their soluble receptors in the serum of SZ patients (see review by Potvin et al., 2008).

Whereas proinflammatory cytokines and their soluble receptors are studied in SZ, the membrane-bound receptors, which are involved in mediating the biological and functions effects of cytokines have not been studied in the blood of SZ patients. Therefore, we examined if abnormality in gene expression of proinflammatory cytokines and their membrane-bound receptors may be associated with schizophrenic pathogenesis. We therefore determined the gene expression of the proinflammatory cytokines, IL-1β, IL-6, and TNF-α, and the cytokine receptors IL-1R1, IL-1R2, IL-1R antagonist (IL-1RA), IL-6R, IL-6 signal transducer (IL-6ST), also known as glycoprotein 130 (Gp130), TNFR1, and TNFR2 in the lymphocytes of SZ patients. To examine if changes in the mRNA levels of these proinflammatory cytokines are also associated with abnormalities of their protein expression levels, as reported by

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some investigators (Munkholm et al., 2013a,b), we determined the protein expression levels of these proinflammatory cytokines in the plasma of SZ patients and NC control subjects.

2. Methods and materials

2.1. Subjects

These studies were conducted in hospitalized patients with SZ (n = 30) admitted to the Psychiatric Clinical Research Center, a part of the General Clinical Research Center, University of Illinois at Chicago. This study was approved by the Institutional Review Board of the University of Illinois at Chicago. All subjects gave informed consent for the study.

After admission to the research unit, patients were kept drug-free up to two weeks before starting treatment. Blood samples were drawn from the patients under a fasting state in the morning. The clinical assessments were performed at the end of the drug-free period before the initiation of treatment.

The comparison subjects were non-hospitalized normal controls (n = 30). Control subjects had no history of psychiatric or major medical disorders. They abstained from any medication for at least two weeks before assessment and blood drawing.

2.2. Clinical assessments

Patients were diagnosed as having SZ according to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* (DSM-IV) criteria, derived by consensus between two trained raters and based on clinical interviews and other available clinical information. Diagnostic and clinical assessments were conducted at admission and at discharge. The discharge diagnosis was considered definitive. Behavioral ratings included scores on the Positive and Negative Syndrome Scale (PANSS).

2.3. Blood processing

Thirty milliliters of venous blood was collected in tubes containing 3.8% (w/v) sodium citrate in DEPC treated water (1 vol:9 vol blood) for plasma. The blood was centrifuged immediately at 210 *g* for 15 min. The platelet-rich plasma (PRP) was removed for platelet isolation. To the red blood cell (RBC) layer, 15 ml of saline was added, mixed gently, and then transferred on Ficoll (2:1 respectively). The sample was then centrifuged at 400 *g* for 40 min. The upper layer above the interface layer was removed and discarded. The interface layer was taken and processed for lymphocyte isolation.

2.4. RNA isolation

Total RNA was extracted from lymphocytes by resuspending the pellet in TRIZOL reagent (Invitrogen, Grand Island, NY, USA,) according to the manufacturer's instructions and treated with DNAse 1 (Invitrogen, Grand Island, NY, USA). The RNA yield was determined by absorbance at 260 nm using NanoDrop®ND-1000 (NanoDrop Technologies, Montchanin, DE, USA). RNA quality was assessed using Agilent Bioanalyzer 2100 (Aligent, Santa Clara, CA, USA). All samples had 28S/18S ratios >1.2 and RNA integrity number (RIN) above 6.6. The mean RIN was 8.1 ± 0.7.

2.5. mRNA determination

Expression levels of mRNA were determined using a two-step real-time RT-PCR (qPCR) method. One microgram of total RNA was reverse transcribed using 50 ng random hexamers, 2 mM dNTP mix, 10 units ribonuclease inhibitor, 50 mM Tris–HCl (pH 8 · 3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 200 units MMLV-reverse transcriptase (Invitrogen) in a final reaction volume of 20 μl. Reverse transcription was performed at 37 °C for 60 min, and enzymes were denatured at 70 °C for 15 min. The cDNA was stored at −20 °C.

Real-time PCR was performed with a MX3005p sequence detection system (Agilent) using pre-designed TaqMan gene expression assays (Applied Biosystems, Grand Island, NY, USA). See Table 1 for details. The stability and optimal number of housekeeping genes were determined using geNORM version 3.4 (PrimerDesign Ltd, Southamptom, UK) according to the manufacturer's instructions (Vandesompele et al., 2002). This comparison identified ACTB and GAPDH as the most stable housekeeping genes. PCR efficiency for all genes, after 5-log dilution series of pooled cDNA, was similar. For each primer/probe set, qPCR reaction was carried out using 10 μl of cDNA (diluted 1:10) in 1× TaqMan Universal PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) per the manufacturer's instructions. Each qPCR plate included a "no reverse transcriptase" and "no template" control to eliminate non-specific amplification and each sample was assayed in triplicate.

For qPCR gene expression analysis, raw expression data (C_t) were normalized to the geometric mean of the two housekeeping genes. Outliers were excluded if the normalized (delta C_t) values were greater than two standard deviations from the group mean. Relative expression levels, reported as fold change, were determined by the $2^{-(\Delta\Delta Ct)}$ method, where ΔΔCT = (CT target − CT normalizer) subject − (CT target − CT endogenous gene) control (Applied Biosystems User Bulletin No. 2). ΔCT values are used for further statistical analysis.

2.6. Determination of plasma protein levels using ELISA

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

2.7. Statistical analysis and effect of confounding variables

We analyzed the data using SAS 9.2 statistical software package. First we used two sample *t*-test to compare NC subjects with SZ patients. In order to examine the effect of confounding variables, we used generalized linear model (PROC GLM in SAS) for each outcome measure to compare those two groups adjusting for fixed covariates like age, sex and race. To examine the association between group and gender we performed a contingency chi-square test. Pearson correlation matrix was used to determine the relationship between the behavioral rating scores and the cytokine mRNA and protein measures.

Table 1
TaqMan primers/probes used for qPCR analysis.

	TaqMan accession	Probe location (exon boundary)	Assay function
ACTB	Hs99999903_m1	1–1	House Keeping (HK)
GAPDH	Hs99999905_m1	3–3	HK
IL-1β	Hs01555410_m1	3–4	Target gene
IL-1RN (IL-1RA)	Hs00893626_m1	4–5	Target gene
IL-1R1	Hs00991010_m1	7–8	Target gene
IL-1R2	Hs00174759_m1	6–7	Target gene
IL-6	Hs00985639_m1	2–3	Target gene
IL-6R	Hs01075666_m1	5–6	Target gene
IL-6ST (Gp130)	Hs00174360_m1	13–14	Target gene
TNF-α	Hs99999043_m1	1–2	Target gene
TNFRSF1A	Hs00533560_m1	1–2	Target gene
TNFRSF1B	Hs00961755_m1	9–10	Target gene