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Original Article

Serum Immunoglobulin G of Neuro-Behçet's Disease Patients Reduce Cerebral Expression Levels of Survival Pathway Factors

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Abstract

Objective: Anti-neuronal antibodies are found in sera of neuro-Behçet's disease (NBD) patients. In this study, our aim was to analyze the potential mechanisms by which NBD immunoglobulin (Ig) Gs affect neuronal dysfunction. **Materials and Methods:** Purified IgGs obtained from pooled sera of six each NBD patients and healthy controls (HCs) were administered to Sprague Dawley rats through intraventricular injection. Control rats received phosphate-buffered saline (PBS) only. Locomotor activity was assessed by open field test on days 0, 10, and 25. Cerebral expression levels of intracellular pathway factors associated with cell survival and viability were measured by real-time polymerase chain reaction. **Results:** Rats treated with NBD IgG exhibited reduced motor activity. On day 25, the mean number of crossings was 44 ± 7, 90 ± 12, and 93 ± 5 and the mean number of rearings was 18 ± 7, 34 ± 5, and 35 ± 6 for NBD IgG, HC IgG, and PBS groups, respectively ($P < 0.001$). Relative expression levels of Akt-1 (0.4 ± 0.2, 1.0 ± 0.3, and 0.9 ± 0.6; $P = 0.004$), DJ-1 (0.6 ± 0.2, 1.0 ± 0.6, and 0.9 ± 0.5; $P = 0.047$), mouse double mininute-2 (0.5 ± 0.3, 0.9 ± 0.2, and 1.0 ± 0.2; $P = 0.002$), and mechanistic target of rapamycin (0.4 ± 0.2, 0.8 ± 0.4, and 0.9 ± 0.6; $P = 0.006$) were significantly lower in NBD-IgG group than HC IgG and PBS groups. By contrast, the expression levels of factors associated with apoptosis (caspase 3, mitochondrial carrier homolog 1, and B-cell lymphoma-2) were comparable among different treatment arms. **Conclusion:** Our results suggest that at least a fraction of NBD IgG interacts with neuronal surface antigens and subsequently decreases neuronal viability through Akt pathway inhibition. By contrast, NBD IgG does not appear to activate neuronal apoptosis. Further identification of the binding sites of serum IgG ıs required.

Keywords: *Akt pathway, Behçet's disease, immunoglobulin G, inflammation, neuro-Behçet's disease*

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Introduction

Behçet's disease (BD) is an auto-inflammatory disorder, characterized with the involvement of multiple systems including mucosal surfaces, blood vessels, joints, the gastrointestinal system, and the brain.[1] Central nervous system involvement, also called as neuro-Behcet's disease (NBD), is characterized by parenchymal lesions in the brainstem and diencephalon and less frequently with dural sinus thrombosis.[2,3] Only a few NBD attacks may result in extreme tissue destruction characterized with significant neuronal loss and atrophy of the afflicted brain regions.[4]

Although parenchymal NBD is considered to be induced by enhanced activity of macrophages, neutrophils, and

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lymphocytes, the presence and impact of antibodies have been increasingly recognized as a putative source of pathogenesis.[5,6] Initially described antibodies were found to be directed against ubiquitously expressed antigens such as heat-shock proteins and apoptosis factors, suggesting a bystander effect of tissue destruction or coincidental cross-reactivity with microorganisms.[7,8] Antibodies interacting with uncharacterized neuronal surface epitopes or specific neuronal antigens (e.g., neurofilament medium) have been recently described in BD and NBD patients.[6,9] These findings have suggested that antibodies may have a causal role in NBD pathogenesis.

In our previous studies, we showed that serum immunoglobulin (Ig) Gs of NBD patients induce neurotoxic effects on SH-SY5Y cells and cause reduced locomotor activity in rats on intraventricular administration.[5,10] Nevertheless, brain samples of NBD-IgG-administered rats do not show gliosis or immune cell infiltration,[5] indicating that harmful effects of IgG are mediated through intracellular pathway dysfunction rather than neuroinflammation. In this study, our aim was to uncover the putative mechanisms, by which NBD IgGs prompt behavioral alterations and neuronal dysfunction. In our previous experiments, SH-SY5Y cells treated with NBD IgG showed altered expression levels of apoptosis and Akt survival pathway factors, suggesting that apoptotic neuronal loss could be one of the factors underlying deleterious effects of NBD IgG.[10] For this reason, we measured mRNA expression levels of several factors associated with cell survival and death in brain samples of rats treated with NBD IgG.

Materials and Methods

Patients and sera

Pooled sera of six randomly recruited parenchymal NBD patients (two women, four men; 36.2 ± 7.1-year old) fulfilling the diagnostic criteria for BD[11] and six age/gender-matched healthy controls (HCs) (three women, three men; 37.5 ± 5.8-year old) were used in animal experiments. Average disease duration of NBD patients was 7.7 ± 2.6 years and the Expanded Disability Status Scale scores ranged between 2.0 and 3.5 (2.7 ± 0.6). During blood sampling, NBD patients were in remission and were not receiving immunosuppressive treatment. None of the patients had the chronic progressive form of NBD or any coexisting disorders. The study was approved by the Institutional Review Board of Istanbul University. All patients gave written informed consent.

IgG purification and rats

IgG was isolated from pooled sera of HCs and NBD patients with a protein A-Sepharose CL-4B column (Sigma, St. Louis, MO). IgG was extracted using 0.05 M citrate buffer, pH 3.0, and neutralized with 1.5 M Tris-buffer, pH 8.8. The IgG solution was then dialyzed against phosphate-buffered saline (PBS) and sterilized with a filter. IgG bands were confirmed by gel electrophoresis. Bradford method was used to measure protein concentrations of purified IgG solutions.

Individually housed male Sprague Dawley rats (8–10 weeks old) were used in animal experiments. Animals were habituated to the experimental room for 1 week before starting the tests. All procedures were conducted in accordance with standard ethical guidelines and approved by the Local Institutional Animal Care and Use Committee.

Injections and motor performance

Cerebroventricular administration of pooled purified IgG from HCs and NBD patients (10 μl/injection of IgG solution with a protein concentration of 10 μg/μl) and PBS (control group) was performed using ten rats for each treatment arm. A cannula (Bilaney Consultants Ltd, Düsseldorf, Germany) was inserted into the right lateral ventricle under ketamine anesthesia. IgG samples were administered by a Hamilton syringe.

Motor performance of rats was assessed by open field test (OFT). Six days after cannula placement, OFT was conducted for baseline (day 0) evaluation. Thereafter, IgG injections were employed every 3 days for 24 days (days 1–24) resulting in administration of eight injections in total. Repeat OFTs were conducted on days 12 and 25. All OFTs were done between 9:00 a.m. and 11:00 a.m. Motor performance was scored by a blinded observer using total number of crossings (horizontal activity) and rearings (vertical activity).

Real-time polymerase chain reaction

The whole brain samples of rats were obtained on day 26 and homogenized. RNA was isolated from homogenates by an RNA isolation kit. Purity and concentration of RNA samples were determined with Thermo Scientific™ NanoDrop 2000. Reverse transcription was performed from 2 μl RNA samples (100 ng/ml) with a commercial kit (Jena Bioscience, polymerase chain reaction [PCR] 511) using Bio-Rad thermal cycler. Real-time PCR was performed with Agilent Stratagene 3005P using PCR master kit (Jena Bioscience), cDNA samples (2 μl), and primers. β-actin, hypoxanthine-guanine phosphoribosyltransferase, and ubiquitin C were used as internal housekeeping control genes. Relative expression levels of genes were calculated by a 2 (− double delta

Table 1: Quantitative polymerase chain reaction primers

Primer name	Sequence 5′-3′
Bcl-2 F	GGTGAACTGGGGGAGGATTG
Bcl-2 R	AGAGCGATGTTGTCCACCAG
Caspase-3 F	TACTCTACCGCACCCGGTTA
Caspase-3 R	CGTACAGTTTCAGCATGGCG
Akt-1 F	GTGGCAAGATGTGTATGAG
Akt-1 R	CTGGCTGAGTAGGAGAAC
DJ-1 F	CGATGTGGTTGTTCTTCCAG
DJ-1 R	GCCGTTCATCATTTTGTCCT
Mdm-2 F	CGGCCTAAAAATGGTTGCAT
Mdm-2 R	TTTGCACACGTGAAACATGACA
mTor F	GGTGGACGAGCTCTTTGTCA
mTor R	AGGAGCCCTAACACTCGGAT
Mtch1 F	GTGGTTGACTCTTACCTGCCT
Mtch1 R	TCTTGTTGGACTCAAGCTGTTT
Beta-actin F*	CCGCGAGTACAACCTTCTTG
Beta-actin R*	CAGTTGGTGACAATGCCGTG
HPRT1 F*	GTCAAGCAGTACAGCCCCAA
HPRT1 R*	TGGCCACATCAACAGGACTC
UBC F*	ACACCAAGAAGGTCAAACAGGA
UBC R*	CACCTCCCCATCAAACCCAA

*Indicates housekeeping genes. HPRT: Hypoxanthine-guanine phosphoribosyltransferase, UBC: Ubiquitin C, Bcl-2: B-cell lymphoma 2, Mdm-2: Mouse double minute 2, mTor: Mechanistic target of rapamycin, Mtch1: Mitochondrial carrier homolog 1, F: Forward, R: Reverse

cycle threshold (ddCT)) method based on the values of PBS-treated brain samples. The primers used in the experiments are shown in Table 1.

Statistics

Multiple group comparisons of behavioral and real-time PCR experiments were performed by ANOVA. $P < 0.05$ was considered as statistically significant.

Results

Administration of neuro-Behçet's disease immunoglobulin G affects motor performance of rats

Intraventricular administration of purified NBD IgG affected the locomotor activity, which was assessed by total number of crossings and rearings. Before the treatment (day 0), the number of crossings ($P = 0.644$) and rearings ($P = 0.251$) for all treatment arms was comparable. After chronic intermittent administration of IgG samples, the number of crossings ($P < 0.001$ for day 12 and day 25) and rearings ($P < 0.001$ for day 12 and day 25) was significantly decreased in the NBD IgG-treated group as compared to other treatment arms. On day 25, the mean number of crossings was 44 ± 7, 90 ± 12, and 93 ± 5 and the mean number of rearings was 18 ± 7, 34 ± 5, and 35 ± 6 for NBD IgG, HC IgG, and PBS groups,

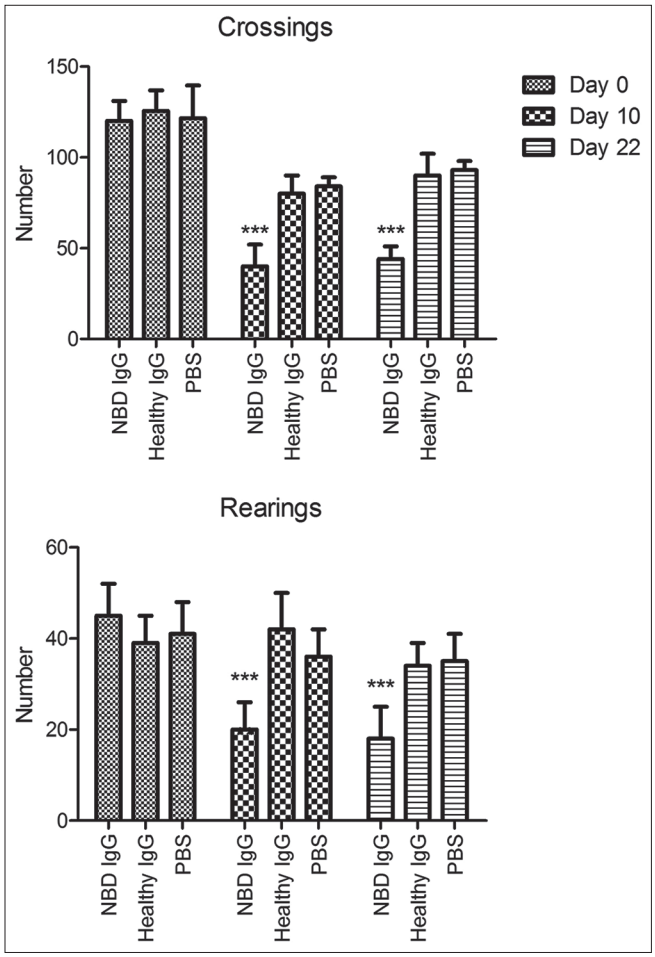

Figure 1: The locomotor activity kinetics of intraventricular immunoglobulin G administered rats in the open field test exemplified by crossing and rearing numbers. NBD: neuro-Behçet disease. ***$P < 0.001$ by ANOVA. Vertical bars indicate standard errors

respectively. There were no significant differences among healthy IgG-treated and PBS-treated groups on days 12 and 25 [Figure 1].

Neuro-Behçet's disease immunoglobulin G administration reduces survival pathway gene expression levels

Brain samples of rats were obtained following chronic intraventricular treatment with serum IgG or PBS, and cerebral expression levels of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt pathway factors were measured by real-time PCR. Expression levels of Akt-1 (0.4 ± 0.2, 1.0 ± 0.3, and 0.9 ± 0.6; $P = 0.004$), DJ-1 (0.6 ± 0.2, 1.0 ± 0.6, and 0.9 ± 0.5; $P = 0.047$), mouse double minnute 2 (Mdm-2) (0.5 ± 0.3, 0.9 ± 0.2, and 1.0 ± 0.2; $P = 0.002$), and mechanistic target of rapamycin (mTor) (0.4 ± 0.2, 0.8 ± 0.4, and 0.9 ± 0.6; $P = 0.006$) were significantly lower in NBD-IgG-treated rats than healthy IgG-and PBS-treated rats, respectively. There were no significant differences among healthy IgG-and PBS-treated rats. Second, the expression levels

of apoptosis-associated factors caspase 3 (0.8 ± 0.4, 1.1 ± 0.6, and 0.9 ± 0.6), mitochondrial carrier homolog 1 (Mtch1) (0.9 ± 0.3, 0.8 ± 0.2, and 1.0 ± 0.3), and B-cell lymphoma 2 (Bcl-2) (0.9 ± 0.6, 0.7 ± 0.1, and 0.9 ± 0.2) were measured. No significant differences could be found among treatment arms (P = 0.668, P = 0.402, and P = 0.411 for caspase 3, Mtch1, and Bcl-2, respectively) [Figure 2].

Discussion

In our previous study, we showed that intracerebral administration of NBD IgG reduced the locomotor activity of rats without affecting measures of anxiety and memory.[5] In this study, which was conducted with sera of different NBD patients, we observed a similar deterioration in motor performance further confirming the neurotoxic action of NBD IgG. Notably, rat brains exposed to NBD-IgG do not show appreciable cellular infiltration or gliosis,[5] suggesting that NBD-IgG affects neuron survival at a subcellular level or induces functional changes only. In line with the first assertion, we observed reduced viability in SH-SY5Y cells treated with NBD-IgG in two previous independent experiments.[5,10]

In this study, to identify the underlying mechanisms of NBD-IgG toxicity, we measured the expression levels of genes associated with cell survival and death (Akt-1, DJ-1, Mdm-2, and mTor) and found significant decrease in the levels of genes regulated by the PI3K/Akt-pathway. Thus, our results suggest that neuronal damage and death induced by *in vitro* or *in vivo* exposure to NBD-IgG might at least partially be related with the dysfunction of this survival pathway.

PI3K/Akt pathway is an intracellular signal transduction pathway that promotes cell survival, growth, and proliferation in response to extracellular signals. Akt also enhances the survival of cells through negative regulation of proapoptotic processes. Inhibition of PI3K or Akt results in decreased cell viability, and thus, PI3K/Akt signaling pathway inhibitors are widely investigated as potential anticancer drugs.[12,13] DJ-1 is a regulator of this pathway and its underexpression leads to decreased phosphorylation-induced activation of Akt and decreased cell survival.[14] Akt promotes the activation of Mdm-2, which, in turn, degrades p53, a proapoptotic oncogene.[15] Another mechanism, by which Akt promotes cell growth, is the activation of mTor.[16] Therefore, reduced cerebral expression of the complex network of these survival-related molecules might be one of the factors lying behind NBD-IgG-induced neuronal death and motor dysfunction. Moreover, mTor is also a critical regulator of autophagy, inhibition of which might lead to the disruption of autophagic lysosome formation and cell death.[16] Interestingly, autophagy has been scarcely investigated in BD, and our understanding on the role of autophagy in BD is limited to the identification of autophagy-related gene polymorphisms in BD patients.[17] Thus, our results prompt further investigation of this important cellular function in BD and NBD patients.

Disease-specific antibodies such as those directed against leucine-rich glioma inactivated-1 (LGI1) have well been demonstrated to decrease cell survival and lead to neuronal death.[18] There are also several reports regarding artificially produced antibodies, which decrease cell viability by the way of reacting with membrane-bound target antigens (e.g., tyrosine-protein kinase receptors, neuropilin-1, toll-like receptors, and GD2 ganglioside), thereby decreasing the expression levels or phosphorylation of intracellular Akt, PI3K, and mTor.[19-23] Conceivably, serum antibodies of NBD

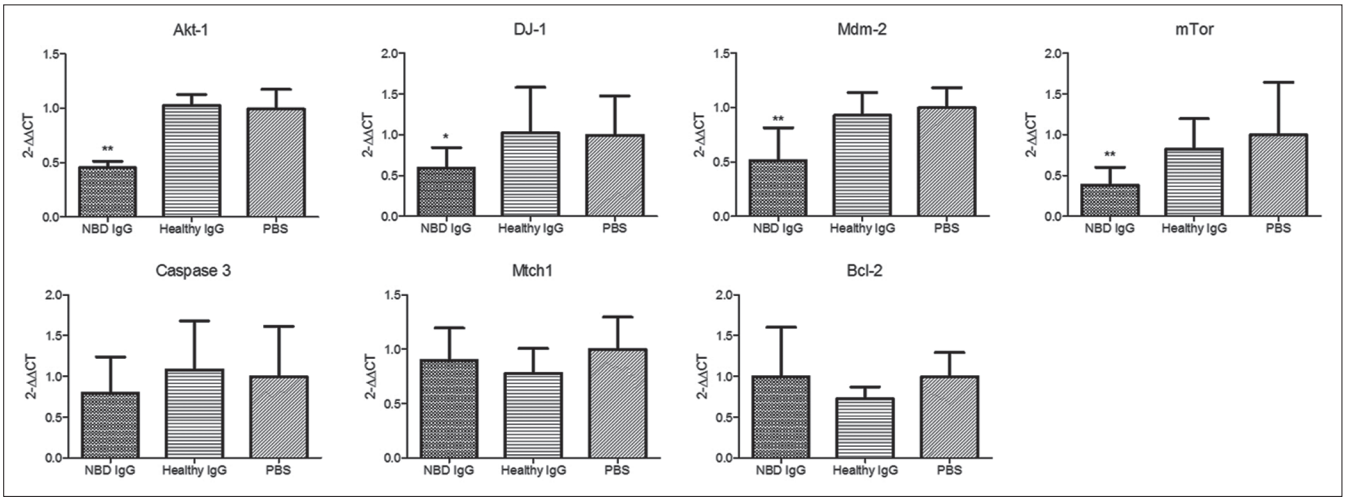

Figure 2: Relative mRNA expression levels of apoptosis and survival pathway factors in brain samples of rats treated with and without intraventricular immunoglobulin G from neuro-Behçet disease patients and healthy controls. *P <0.05; **P <0.01 by ANOVA. Vertical bars indicate standard errors

patients might harbor serum antibodies interacting with similar neuronal cell surface antigens that regulate PI3K/Akt pathway factors.

Another important finding was the absence of alteration in cerebral expression levels of major apoptosis-associated genes such as caspase 3, Mtch1, and Bcl-2. Mtch1 expression levels were of particular interest since this protein mediates apoptosis through Bcl2-independent and mitochondria-dependent mechanisms and NBD patients display increased anti-Mtch1 antibody positivity.[24,25] Furthermore, our results contradicted a previous study, which showed enhanced expression levels of Akt pathway and apoptosis genes (Bax, Bcl-2, caspase 3, caspase 9, Akt-1, mTor, and DJ-1) in SH-SY5Y neuroblastoma cells treated with serum IgGs of NBD patients.[10] A likely explanation of this discrepancy could be that healthy brain neurons and neuroblastoma cells yield diverse expression patterns to IgG exposure. Alternatively, the enhanced apoptosis and Akt pathway expression might represent the hyperacute response (72 h) of neurons to NBD IgG treatment, whereas the expression results obtained from the present *in vivo* study could be representative of the late response to 24 days of chronic NBD IgG treatment. In between these two time points, neuronal apoptosis might be averted via compensating counter measures.

A limitation of our study was the absence of control IgGs obtained from BD patients without neurological symptoms and from other inflammatory and noninflammatory neurological diseases. In future studies, it might be important to know whether the observed expression results are specific to NBD or not and whether this intriguing action of antibodies can be used as a diagnostic method. Likewise, impact of antibodies on the expression patterns of other organs afflicted in BD (e.g., gastrointestinal system, uvea, skin, and blood vessels) should also be investigated. Moreover, testing the neurotoxic effects of individual sera rather than using pooled sera might also disclose potential differences of antibody pathogenicity among NBD patients.

Conclusion

Overall, our NBD IgG results obtained in three independent experiments indicate the neurotoxic effects of NBD IgG. Putatively, this effect appears to be elicited through the interaction of serum antibodies with certain cell surface antigens, which regulate intracellular signaling pathways involved in cell viability, apoptosis, and autophagy. It is well known that certain brain regions such as the brainstem and diencephalon are more preferentially afflicted in NBD.[26] Even though the antibody-mediated mechanism described in our study might not be the genuine culprit of the severe focal brainstem atrophy observed after NBD attacks, it might at least enhance susceptibility to neuron death induced by auto-inflammation. Better characterization of the target antigens of these antibodies might pave the way toward patient-specific treatment methods for NBD and aid in the selection of NBD patients, who could potentially be responsive to antibody-depleting treatment methods such as plasma exchange.

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Conflicts of interest

There are no conflicts of interest.

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