Impact of Neuro-Behçet Disease Immunoglobulin G on Neuronal Apoptosis

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ABSTRACT

Introduction: Parenchymal neuro-Behçet disease (NBD) is encountered in 5%–15% of Behçet disease (BD) patients and is characterized by inflammation of the brainstem and diencephalon structures. Neuronal apoptosis has been shown to participate in neuronal cell loss. Anti-neuronal antibodies have been identified in NBD patients. However, the pathogenic properties of these antibodies have not been studied.

Methods: To delineate the potential pathogenic activity of serum antibodies on neurons, pooled sera from seven NBD patients and seven healthy controls were divided into purified immunoglobulin G (IgG) and IgG-depleted serum fractions, and each fraction was administered to cultured SH-SY5Y neuroblastoma cells. Cell death was evaluated with a toxicity assay and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Moreover, expression levels of several apoptosis markers were evaluated with real time polymerase chain reaction (PCR).

Results: Administration of NBD IgG to cultured SH-SY5Y cells induced significantly increased cell death and apoptosis compared with other treatments. NBD IgG also enhanced the mRNA expression levels of major apoptosis and cell survival pathway factors.

Conclusion: Our results suggest that IgGs isolated from the sera of NBD patients have a neurotoxic activity that is presumably mediated by apoptotic mechanisms.

Keywords: Behçet disease, neuro-Behçet disease, immunoglobulin G, terminal deoxynucleotidyl transferase dUTP nick end labeling, neurotoxicity

INTRODUCTION

Behçet disease (BD) is an inflammatory disorder that is characterized by recurrent oral aphthae, genital ulcerations, and uveitis. In addition, other organs, including the central nervous system (CNS), might be affected in BD patients (1). BD with CNS involvement, also known as neuro-BD (NBD), is encountered in 5%–15% of BD patients in parenchymal or vascular forms. Parenchymal NBD is most typically characterized by inflammatory lesions that extend from the brainstem to the thalamus, diencephalon, basal ganglia, and internal capsules (2,3).

Parenchymal NBD is generally caused by large brain lesions with infiltrating macrophages, neutrophils, and lymphocytes, and neuronal apoptosis appears to be an important contributor to neuronal destruction in these lesions (1,2,3). However, many BD patients with no demonstrable brain lesions develop neurological symptoms (4,5). Anti-neuronal antibodies might putatively cause these symptoms in the absence of evident brain inflammation. Although antibodies to various heat-shock proteins, neuronal membrane antigens, and apoptosis factors have been identified in BD and NBD patients, the pathogenic significance of these antibodies has never been studied (6,7,8,9).

To demonstrate the potential pathogenicity of these antibodies, serum immunoglobulin G (IgG) and IgG-depleted sera of NBD patients were administered to cultured SH-SY5Y neuroblastoma cells, and the potential neurotoxicity of anti-neuronal IgG from NBD patients was analyzed by in vitro studies.

METHODS

Sera and Anti-Neuronal Antibody Assays
The sera of seven parenchymal NBD patients who fulfilled the diagnostic criteria for BD (10) and those of seven age-/gender-matched healthy controls were used. The sera were frozen at −80°C until assayed. The study was approved by the Ethics Committee of Istanbul Faculty of Medicine of Istanbul University. Informed consent was obtained from all participants.
IgGs were isolated from pooled healthy and NBD sera using a protein A–sepharose CL-4B column (Sigma, St. Louis, MO), as previously reported (11). In brief, 1 ml of serum was incubated with 0.5 ml of protein A–sepharose for 2 h at room temperature (RT). After removing the serum, IgG was extracted using 0.05 M citrate buffer at pH 3.0 and immediately neutralized with 1.5 M Tris-buffer at pH 8.8. The IgG-containing solution was then dialyzed against phosphate-buffered saline (PBS) and filter sterilized. To obtain IgG-depleted serum, the serum was recovered from the protein A column by centrifuging the column for 2 min at 500 rpm. The recovered serum was reabsorbed on a protein A–sepharose column for 2 h at RT and recovered by centrifugation. Each serum was reabsorbed four times. IgG depletion in the reabsorbed sera was confirmed by the absence of IgG bands in the expected molecular weight range (45–55 kDa for heavy chains and 25–35 kDa for light chains) by gel electrophoresis. Protein concentrations of purified IgG solutions were measured using the Bradford method.

**Neuroblastoma Cell Line and Toxicity Assay**

The neuroblastoma cell line (SH-SY5Y) (ATCC, Wesel, Germany) was grown in minimal essential medium with F12-nutrient mixture (MEM/F-12), supplemented with non-essential amino acids (Sigma-Aldrich, St. Louis, MO, USA). A toxicity assay was performed to assess growth inhibitory and cytolytic effects of serum and serum IgG on neuroblastoma cells in culture. IgG-depleted sera of NBD and healthy control groups were diluted four times with culture medium and filter-sterilized. Likewise, purified IgG solutions (10 µg/µL protein concentration) from NBD patients and healthy controls were diluted four times with culture medium and filter sterilized. To obtain IgG-depleted serum, the serum was recovered from the protein A column by centrifuging the column for 2 min at 500 rpm. The recovered serum was reabsorbed on a protein A–sepharose column for 2 h at RT and recovered by centrifugation. Each serum was reabsorbed four times. IgG depletion in the reabsorbed sera was confirmed by the absence of IgG bands in the expected molecular weight range (45–55 kDa for heavy chains and 25–35 kDa for light chains) by gel electrophoresis. Protein concentrations of purified IgG solutions were measured using the Bradford method.

**Terminal Deoxynucleotidyl Transferase dUTP Nick end Labeling (TUNEL) Staining**

The apoptosis intensity of SH-SY5Y cells treated with NBD patients’ or healthy individuals’ IgG and serum samples was estimated by TUNEL staining as per the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN, USA).

**Real Time Polymerase Chain Reaction (PCR)**

Total RNA was obtained from SH-SY5Y cells using TRIzol and reverse transcribed into complementary DNA with a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA USA). Quantitative PCR was performed using 2 µL of cDNA, 1 µL ddH2O, 5 µL SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA USA), and 1 µL of forward and reverse primers (Table 1). All samples were studied in triplicate and the mRNA abundance was normalized for each sample to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT-PCR and melting curve analysis were performed with a LightCycler 480 II (Roche Diagnostics, Mannheim, Germany) instrument. Data were analyzed according to the ∆∆Ct method and the results were expressed as relative mRNA levels.

**Statistical Analysis**

Multiple group comparisons were performed using the ANOVA multiple comparison test and Tukey’s post-hoc test. A p value of <0.05 was considered statistically significant.

**RESULTS**

Clinical and Demographic Features of Patients

Pooled sera of seven NBD patients (one woman and six men; mean age±standard deviation, 37.2±4.1) and those of 7 seven age-/gender-matched healthy controls were used for all in vitro studies. The average disease duration of NBD patients was 6.9±2.1 years, and the Expanded Disability Status Scale scores ranged between 2.0 and 4.5 (2.7±0.9). During blood sampling, NBD patients did not experience an attack and were not under immunosuppressive treatment.

**NBD IgG Administration Increases Neuronal Cell Death and Alters Apoptosis Parameters**

Counts of SH-SY5Y cells were significantly reduced in NBD-IgG-treated wells compared with other treatment arms at 72 h of incubation time (p<0.05 by ANOVA). In NBD-IgG-treated wells, an average of approximately 40% reduction was observed in total cell counts (Figure 1).

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Table 1. Primers used in real time polymerase chain reaction (PCR) analysis of SH-SY5Y cells

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Forward primer sequence (5'-3')</th>
<th>Reverse primer sequence (3'-5')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>TGGAGCTGCAGAGATGTGATGTA</td>
<td>GAAGTTGCCGTCAGAAAACATG</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>CATGTGTTGGAGAGCGGTCA</td>
<td>TCACCTTGAGCCAGATAGG</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>ATTTGGAATTGGATCGTGA</td>
<td>GGCAAGGCTGAATAAATTGAA</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>GCTGTTCAGGCCCATATGAT</td>
<td>GAGCTACGCGCAAGATTGCA</td>
</tr>
<tr>
<td>Akt-1</td>
<td>CTCATTGGCGGTATCGT</td>
<td>CTGCGCGGAGTAGGAGACTG</td>
</tr>
<tr>
<td>mTOR</td>
<td>CTCACTACATATATATAAGC</td>
<td>GTGTTCACTTTTGTCTGAT</td>
</tr>
<tr>
<td>DJ1</td>
<td>GGAGACGGTCATCCGTGTA</td>
<td>TACACAGCAGCAGACTCGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAAGGTTGAAGAGTGCGGAGTA</td>
<td>GACAAGCTTCCCGGTTCAG</td>
</tr>
</tbody>
</table>

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; mTor: mechanistic target of rapamycin.
Two-group comparisons were significant for NBD-IgG-treated cells versus other treatment arms (p<0.05 for all by Tukey’s post hoc analysis). There were no significant differences between other treatment arms in terms of cell counts.

To evaluate the involvement of apoptosis in neuronal cell loss, TUNEL staining was performed for SH-SY5Y cells treated with the same treatment arms. Only cells treated with NBD IgG showed an abundance of apoptotic cells (4–7 cells in each ×10 microscope field). In cells treated with healthy control serum, healthy control IgG, NBD serum, or culture medium, much fewer apoptotic cells were observed (<1 cell per ×10 microscope field) (Figure 2). These results suggested that NBD IgG (but not IgG-depleted sera of NBD patients) reduces neuronal survival by enhancing apoptosis.

**NBD IgG Administration Enhances Apoptotic and Anti-Apoptotic Factor Expression Levels**

To analyze the impact of NBD IgG and serum samples on neuronal apoptosis and survival pathway factor expression, mRNA levels of a panel of intracellular apoptosis factors were measured. NBD-IgG-treated cells (for 72 h) demonstrated significantly increased mRNA levels of pro-apoptotic Bax, caspase 3, and caspase 9 compared with cells treated with healthy control serum, healthy control IgG, NBD serum, or culture medium (p<0.01 for all three parameters with ANOVA). Notably, expression levels of anti-apoptotic Bcl-2 were also increased in SH-SY5Y cells treated with NBD IgG but not in those treated with other treatment (p<0.001 with ANOVA). Moreover, expression levels of the anti-apoptotic components of the phosphoinositide 3-kinase (PI3K)/Akt pathway Akt-1, mechanistic target of rapamycin (mTOR), and DJ1 were increased in NBD-IgG-treated cells compared with other treatment groups (p<0.01 for mTOR and DJ1 and p<0.001 for Akt-1 by ANOVA) (Figure 3). Two-group comparisons were significant for NBD-IgG-treated cells versus other treatment arms (p<0.01 for all by Tukey’s post hoc analysis). There were no significant differences between other treatment arms in terms of mRNA expression levels.

**DISCUSSION**

In our study, with multiple independent methods, we showed that serum NBD IgG has a neurotoxic activity and effectively prevents the proliferation of neurons. Our real time PCR data indicated that...
the neurotoxic activity could be because of an interference with intracellular apoptosis and survival pathways. Moreover, the absence of any neurotoxic activity with IgG-depleted serum indicates that IgG, but not any other humoral factor (e.g., cytokines and granzyme), is involved in this toxic effect. Only a few reports have demonstrated the beneficial effects of antibody-depleting treatment methods (e.g., plasma exchange and intravenous immunoglobulin) in BD patients (12,13). The demonstration of neurotoxic IgG in our study supports this notion and suggests that these treatment methods also work for NBD.

Apoptotic neuronal death is a remarkable pathological finding observed in brain lesions of NBD patients. Unlike other neuroinflammatory disorders, extensive neuronal loss is observed in NBD lesions, in addition to T-cell, B-cell, and monocyte infiltration (14). Although pro-apoptotic cytokines, such as IL-6, have been implicated in neuronal apoptosis, this view has not been supported with experimental data (14). Our results suggest that serum IgG is the real culprit for this phenomenon.

The PI3K/Akt pathway is a major survival pathway and is notably located within the apoptosis, autophagia, and mitochondrial pathways (15). Akt-1, mTor, and DJ1, factors of the PI3K/Akt pathway, have anti-apoptotic activity, and in animal models of neurotoxicity, the reduced expression of these molecules has been associated with increased neuronal loss (16,17). In several clinical and experimental studies, the activation of the PI3K/Akt pathway has been shown to lead to increased anti-apoptotic bcl-2 gene expression and subsequently, reduced caspase gene expression (15,16,17).

In line with the toxicity and TUNEL staining assays, NBD-IgG-treated SH-SY5Y cells showed increased mRNA expression levels of pro-apoptotic factors, Bax, caspase 3, and caspase 9. However, notably, expression levels of anti-apoptotic factors, Bcl-2, Akt-1, mTor, and DJ1, were also increased in NBD-IgG-treated cells. These results indicate that the anti-apoptotic and survival pathways are activated as a compensating measure to overcome the toxic activity of NBD IgG. In several previous reports, the compensating increased Akt-1 expression has been documented (18,19). Similar to our experimental setting, this increase has not been effective in preventing cell death.

In conclusion, our study demonstrated for the first time the neurotoxic and pro-apoptotic activity of NBD IgG. These findings not only shed new light on NBD pathogenesis but also emphasize the potential benefits of antibody-depleting treatment methods for NBD patients. Better understanding of the apoptosis pathways involved in NBD-related neuronal loss in future studies might lead to the innovation of novel apoptosis-inhibiting treatment methods for this potentially disabling neurological inflammatory disease.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Istanbul University Istanbul School of Medicine.

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Peer-review: Externally peer-reviewed.


Conflict of Interest: No conflict of interest was declared by the authors.

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nyl-1,2,3,6-tetrahydropyrindine (MPTP) and oxidative stress. Proc Natl Acad Sci U S A 2005; 102:5215-5220. [CrossRef]


