International Immunopharmacology 14 (2012) 550–557

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Effects of intravenous immunoglobulin on alpha synuclein aggregation and neurotoxicity

Lynnae M. Smith [a], Andrea C. Klaver [a], Mary P. Coffey [b], Loan Dang [c], David A. Loeffler [a,*]

[a] Department of Neurology Research, William Beaumont Hospital Research Institute, 3811 West Thirteen Mile Road, Royal Oak, MI 48073, USA
[b] Department of Biostatistics, William Beaumont Hospital Research Institute, 3811 West Thirteen Mile Road, Royal Oak, MI 48073, USA
[c] Eye Research Institute, Oakland University, 2200 Squirrel Road, Rochester, MI 48309, USA

ARTICLE INFO

Article history:
Received 5 April 2012
Received in revised form 11 September 2012
Accepted 12 September 2012
Available online 29 September 2012

Keywords:
Alpha-synuclein
Antibodies
Intravenous immunoglobulin
Oligomers
Thioflavin-T
Western blots

ABSTRACT

α-Synuclein is thought to contribute to the pathogenesis of Parkinson's disease (PD). It is the main protein in Lewy bodies, the pathognomonic inclusion bodies in the PD substantia nigra, and mutations which increase its aggregation and/or expression are associated with familial early-onset parkinsonism. Soluble oligomers are considered to be α-synuclein's most neurotoxic conformation. We previously reported that intravenous immunoglobulin (IVIG) products contain specific antibodies to α-synuclein which do not prevent development of four-day α-synuclein oligomers. The objective of this study was to further examine IVIG's effects on α-synuclein's aggregation and neurotoxicity. The IVIG product Gammagard (Baxter Healthcare) did not prevent the development of nine-day α-synuclein oligomers, nor did it degrade preformed oligomers, as shown by western blots performed on gels run under reducing/denaturing conditions and native gels. In western blots of native gels, an additional low molecular weight band (~22 kDa) was detected in α-synuclein incubated for four days in Gammagard, but not in Gammagard alone. No significant differences were found for Thioflavin-T reactivity between α-synuclein amorphous aggregates grown in Gammagard vs. those grown in phosphate-buffered saline. Gammagard partially protected SK-N-BE(2)M17 human neuroblastoma cells against α-synuclein oligomer toxicity ($p = 0.007$ vs. protective effects of normal human IgG). These findings suggest that although IVIG does not prevent α-synuclein aggregation, it still may reduce α-synuclein neurotoxicity through an unknown mechanism.

1. Introduction

α-Synuclein is a 140 amino acid protein located primarily in presynaptic terminals of CNS neurons which has been implicated in the pathogenesis of Parkinson's disease (PD) [1–4]. Increased α-synuclein expression (due to its gene duplication and triplication) and mutations which increase its aggregation are associated with autosomal dominant early-onset parkinsonism [5]. α-Synuclein mRNA is also increased in the midbrain, and in melanized dopamine midbrain neurons, in individuals with sporadic PD [6,7]. It is the major protein in Lewy bodies, the intracytoplasmic inclusions in the PD substantia nigra. Although Lewy bodies contain fibrillar α-synuclein, soluble oligomers are thought to be the most neurotoxic conformation of the protein [8–10]. The significance of Lewy bodies is unclear, with some investigators suggesting that they may be neuroprotective rather than neurotoxic [11]. Both neuroprotective and neurotoxic roles have also been hypothesized for soluble α-synuclein, depending on its concentration [12]. Therapeutic targeting of α-synuclein has focused primarily on inhibiting its aggregation [13–16]. In addition to its direct neurotoxicity, α-synuclein may indirectly promote neuronal loss by increasing inflammation in the PD brain through microglial activation [17], increased expression of proinflammatory cytokines [18], and/or complement activation [19].

α-Synuclein is secreted by neurons, therefore it is detectable extracellularly as well as within neurons [20,21]. α-Synuclein levels in plasma and CSF have been suggested as potential biomarkers for PD [22–25], although the specificity of alterations in CSF α-synuclein for PD has been questioned [26,27]. Recent studies indicate that α-synuclein can be transferred between neurons [28–30], suggesting prion-like behavior [31]. Extracellular α-synuclein is therefore a prime therapeutic target in PD and the other synucleinopathies, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). Increasing brain anti-α-synuclein antibody levels may be a useful approach for treating these disorders, as suggested by studies in which vaccination with α-synuclein [32] or administration of anti-α-synuclein antibodies [33] reduced neuronal accumulation of α-synuclein and synaptic loss in transgenic mice expressing human α-synuclein.

* Corresponding author at: William Beaumont Hospital Research Institute, 3811 West Thirteen Mile Road, Royal Oak, MI 48073, USA. Tel.: +1 248 551 3170; fax: +1 248 551 5069.
E-mail addresses: Lynnae.Smith@beaumont.edu (L.M. Smith), Andrea.Klaver@beaumont.edu (A.C. Klaver), Mary.Coffey@beaumont.edu (M.P. Coffey), dang@oakland.edu (L. Dang), DLoeffler@beaumont.edu (D.A. Loeffler).

1567-5769/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2012.09.007

We previously reported the presence of antibodies to α-synuclein monomer and soluble oligomers in intravenous immunoglobulin (IVIG) preparations [34]. These products consist of purified plasma immunoglobulins from thousands of healthy subjects and are used, often off-label, to treat many autoimmune, infectious, and idiopathic disorders [35]. Although IVIG's benefits in some of these disorders are likely to be due in part to its anti-inflammatory actions [36], its mode of action is often unclear. In pilot studies, IVIG appeared to stabilize cognitive functioning in individuals with Alzheimer's disease (AD) [37,38], and a phase III IVIG trial with AD patients is in progress. IVIG administration to patients with DLB and MSA has also been reported [39,40] but little is known of its effects on α-synuclein. In our previous study [34], as shown by western blots of reducing/denaturing gels, IVIG preparations did not prevent short-term (four-day) development of α-synuclein soluble oligomers, although alterations were seen in the distribution of the oligomer bands. The objectives of the present study were to further examine the effects of IVIG on α-synuclein aggregation, by determining its effects on the formation of longer-term (nine-day) α-synuclein soluble oligomers and amorphous aggregates as well as preformed α-synuclein oligomers, and to determine its possible protective effects against α-synuclein oligomer neurotoxicity. The IVIG preparation Gammagard (Baxter Healthcare) was chosen for this study because in our earlier investigation [34] it appeared to contain the highest specific antibody levels to α-synuclein monomer among three IVIG preparations examined.

2. Methods

2.1. α-Synuclein disaggregation

Recombinant human α-synuclein (0.5 mg; rPeptide, Bogart, GA) was disaggregated by resuspending in 0.25 ml trifluoroacetic acid (TFA; Sigma-Aldrich, Inc., St. Louis, MO) followed by an equal volume of hexafluoro-2-propanol (Sigma-Aldrich). After water bath sonication for 1 h, it was aliquoted into 0.6 ml eppitubes (20 μl/tube), air-dried overnight in a fume hood, and stored at −20 °C.

2.2. Production of α-synuclein monomer and soluble oligomers

Soluble oligomers of α-synuclein were produced by resuspending two eppitubes of previously disaggregated α-synuclein in a total of 5 μl of phosphate buffered saline (PBS, 0.01 M, pH 7.2). An additional 50.3 μl of PBS was then added and the preparation was equally divided between two tubes, and then incubated in a shaking water bath at 37 °C for four or nine days. The protein concentration of this preparation, measured with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), was 43 μg/ml (3 μM). To produce monomeric α-synuclein, used as a control in western blot studies, one eppitube of disaggregated α-synuclein was resuspended in 0.6 ml HPLC-grade water whose pH had been adjusted to 3.0 with TFA. This was repeated twice more, yielding 1.8 ml of α-synuclein. 21.8 mg of Tris base (Trizma base, Sigma-Aldrich) was added to bring the Tris concentration to 100 mM. The pH of this solution was adjusted to 8.8 by adding 3.8 μl of 12.1 N HCl. This preparation, whose protein concentration was measured to be 1 μg/ml, was centrifuged (12,000 rpm [11,752×*g*]×5 min at room temperature), passed through a 0.2 μm filter (GHP Acrodisc 13 mm Syringe Filter with 0.2 μm GHP Membrane, Pall Life Sciences, East Hills, NY), and used immediately.

2.3. IVIG

Gammagard Liquid (Immune Globulin Intravenous [Human]) 10% (Baxter Healthcare Corp., Westlake Village, CA) was used in this study.

2.4. Effects of IVIG on development of α-synuclein soluble oligomers: western blots

α-Synuclein was resuspended to 43 μg/ml (3 μM) in PBS, in Gammagard (1:100 dilution in PBS; final concentration = 1 mg/ml), or, as a negative control, in undiluted human myeloma proteins (HMP; human IgG1 kappa chain, The Binding Site, San Diego, CA, cat. # BP078; concentration = 1 mg/ml). (HMP are spontaneously arising monoclonal antibodies found in sera of patients with lymphoproliferative disorders such as multiple myeloma and Waldenstrom's macroglobulinemia [41]; their antigenic specificity is generally unknown. Using our previously published ELISA [34], we did not detect anti-α-synuclein antibodies in HMP [data not shown].) These preparations were then incubated for nine days in oligomer-promoting conditions as described above, and then evaluated by gel electrophoresis with western blotting in three experiments using PBS alone as a negative control. To allow comparisons with our previous study, western blots were also performed on α-synuclein incubated for four days in these same conditions. 24 μl of each α-synuclein oligomer preparation (1.03 μg) was mixed with an equal volume of Laemmli sample buffer containing 5% 2-mercaptoethanol (both from Bio-Rad), boiled for 5 min, and then loaded into individual lanes of 4–20% Precise Protein Gels (Pierce Protein Research Products, Thermo Fisher Scientific, Rockford, IL). Molecular weight standards were Pierce Blue Prestained Protein Molecular Weight Marker Mix (Thermo Fisher Scientific). After electrophoresis, the proteins were transferred to Westran S PVDF membranes (Whatman International Ltd., Maidstone, UK). Membranes were blocked with Blocking Buffer for Near Infra Red Fluorescent Western Blotting (Rockland Immunocytochemicals, Gilbertsville, PA) overnight at 4 °C, followed by incubation in mouse monoclonal anti-α-synuclein antibody clone syn 211 (Santa Cruz Biotechnology; 1:200 dilution) for 2 h at room temperature, and then Mouse IgG (H&L) Antibody IRDye800® Conjugated Pre-Adsorbed Rabbit Polyclonal (Rockland Immunocytochemicals; 1:15,000 dilution) for 1 h at room temperature. Bands were visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE).

To determine the origin of additional bands that were seen in these experiments when α-synuclein oligomers were developed in the presence of Gammagard (see Results), additional western blots were performed on gels containing Gammagard (1:100 dilution) alone, HMP alone, and PBS alone, and on gels containing each of the experimental conditions (α-synuclein oligomers grown in Gammagard, HMP, and PBS, as well as Gammagard, HMP, and PBS alone) with omission of the primary antibody during western blot staining. Normal mouse IgG (clone MOPC-21, Sigma-Aldrich Corp., St. Louis, MO) was used as an additional negative control in these experiments, which were performed on two occasions.

Western blots were performed on native gels on three occasions. α-Synuclein oligomers were generated in PBS, Gammagard (1:100 dilution), or HMP for four days, and then these preparations were diluted 1:4 in PBS. 7.5 μl of the diluted α-synuclein preparations (0.08 μg) was combined with 2.5 μl of sample buffer, and then electrophoresed through NativePAGE™ Novex® 4–16% Bis–Tris Gels (Invitrogen/Life Technologies, Grand Island, NY). Gammagard (1:100) alone, HMP alone, and PBS were electrophoresed in additional lanes. The molecular weight ladder was NativeMark™ Unstained Protein Standard and the sample buffer was NativePage™ 4× Sample Buffer (both from Invitrogen/Life Technologies); the blocking buffer was Odyssey® Blocking Buffer (LI-COR Biosciences). Bands were again visualized with LI-COR's Odyssey Infrared Imaging System.

2.5. Effects of IVIG on preformed α-synuclein oligomers: western blots

α-Synuclein oligomers were produced by incubating α-synuclein for four days in PBS as described above, and then an equal volume of Gammagard (1:100 dilution in PBS), HMP, or PBS was added.