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Longitudinal Effects of Intravenous Immunoglobulin on Alzheimer's Cerebrospinal Fluid Proteome

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Abstract

Intravenous immunoglobulin (IVIg) therapy has shown promise in the treatment of Alzheimer's disease (AD). In this study, serial cerebrospinal fluid (CSF) samples from a group of subjects with AD undergoing IVIg immunotherapy are analyzed to identify IVIg-related changes. CSF samples from eight subjects were collected before therapy, after six months of therapy, and after a three month drug washout period. Samples were analyzed using a gel-based proteomics strategy and IVIg-related changes were determined by gel spot % volumes. An initial assessment of the data revealed consistent and considerable change in 69 spots. A statistical analysis revealed 79 protein spots with a significant change after six months; furthermore, in a subset of these (25), the % volume change was either maintained or reversed in the washout samples. The proteins that showed a significant change during IVIg therapy, including Ig molecules, gelsolin, transferrin, and transthyretin, have been previously implicated in AD. This study provides preliminary findings regarding a group of CSF proteins that may be associated with the treatment of AD, as well as the potential use of IVIg as an AD immunotherapy.

Keywords

Alzheimer's disease; Biomarker; Cerebrospinal fluid; Intravenous immunoglobulin

1 Introduction

Alzheimer's disease (AD) is the leading cause of dementia in the elderly and affects over 5.3 million people in the United States [1]. The disease is characterized by amnesic type memory impairment and deterioration of language skills, both of which progressively worsen over the course of the disease [2–4]. Histopathologically, AD is characterized by

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Conflicts of interest

The authors declare no conflict of interest.

intracellular tangles of tau protein and extracellular plaques that are primarily composed of amyloid beta (A β) [5].

Active immunization against A β in preclinical studies using transgenic animal models of AD resulted in the reduction of A β in both cerebrospinal fluid (CSF) and plaque burden [3–4]. The change in A β plaque burden was also associated with restored cognitive function in some transgenic animals [5–6]. Similar results were obtained in animal studies with passive immunization using monoclonal antibodies against A β [7].

One approach to passive immunization is the use of intravenous immunoglobulin (IVIg) therapy. IVIg is a biological product that contains intact IgG molecules with a distribution of IgG subclasses equivalent to that in normal human serum [8]. After an IVIg infusion of 2 g/kg (a typical dose used to treat other neurological diseases), serum IgG levels are found to increase five-fold. Serum levels return to baseline after 21 to 28 days. IgG levels in CSF are found to increase two-fold over the first 48 hours and then return to normal within a week [9].

When subjects with various neurological disorders were given IVIg infusions, a significant increase in anti-A β antibodies in both CSF and serum was found. In CSF, the total amount of A β , and the amount of A β _{1–42} specifically, was shown to significantly decrease after IVIg infusions. In serum, however, the total amount of A β significantly increased after IVIg infusions [10]. There are several proposed molecular-level effects of the anti-A β antibodies in AD subjects. The first is that the antibodies bind to the aggregated A β and recruit microglia to phagocytose the aggregates [11]. The second proposed effect is that the anti-A β antibodies provide a peripheral sink for A β causing the A β to leave the CNS and enter the plasma where it is degraded [4,12]. Lastly, there is evidence that anti-A β antibodies prevent A β aggregation and may be able to break up already formed aggregates [13–14].

Dodel *et al.* reported results from a pilot study with five AD subjects who were given 0.4 g/kg IVIg every four weeks over the course of six months [15]. They reported that the CSF concentration of total A β decreased by 30% and the serum concentration increased by 233%. Based on Mini-Mental State Examination (MMSE) scores before and after six months of IVIg treatment, none of the five subjects showed any cognitive decline, and several showed an improvement [15].

In an open label safety and dose-ranging study, subjects with moderate AD were treated with IVIg for six months, followed by a three month washout period, and then treatment resumed for another nine months [16]. As a result of this therapy, CSF A β decreased significantly after six months of therapy, returned to baseline after IVIg washout and decreased again after IVIg treatment resumed. MMSE scores increased an average of 2.5 points after six months, returned to baseline during washout, and remained stable during subsequent IVIg therapy. An analysis of the longitudinal changes in CSF protein expression may provide insights into the disease modifying effects of IVIg treatment.

In this report, serial CSF samples collected from these AD subjects undergoing IVIg immunotherapy [16] are analyzed using 2DE to characterize the potential effects of IVIg treatment. Longitudinal study designs benefit from sampling strategies where individuals

serve as their own biological control. CSF samples taken before therapy, after six months of therapy, and after a three month IVIg washout, were used for this study. After an initial assessment of the data, two separate statistical analyses were then used to identify 1) proteins with significant concentration changes with the therapy, followed by 2) those with sustained or reversed changes during the washout. These changes identify possible surrogate endpoints of AD therapy and give information about the IVIg therapeutic mechanism of action in AD subjects. This study is the first to report on the analysis of serial CSF samples from AD subjects to monitor therapy-induced changes in the CSF proteome.

2 Materials and methods

2.1 Cerebrospinal fluid samples

With institutional review board approval, lumbar CSF samples were obtained from eight subjects enrolled in a phase I clinical trial of IVIg therapy for the treatment of AD at Weill Cornell Medical College [16]. All subjects have been diagnosed with probable AD based on NINCDS-ADRDA criteria [17]. The subjects have been randomly assigned to one of four different dosing regimens: 0.4 g/kg every two weeks, 0.4 g/kg every week, 1 g/kg every two weeks, and 2 g/kg every month. Serial lumbar CSF samples taken before IVIg therapy (t=0), after six months of therapy (t=6), and after three months of IVIg washout (t=9) were analyzed for this study. All samples were collected by the same trained individual using the same protocol across all subjects and timepoints. This information is summarized in Table 1. For the washout period, a CSF sample from subject #5 was not available. CSF samples and their corresponding 2DE gels appeared free from blood contamination. Samples were stored at -70°C until used.

2.2 Two-dimensional gel electrophoresis

Proteins in the CSF samples were separated and resolved using 2DE. Each sample was run twice. The details of the 2DE protocol have been previously published [18]. Briefly, for each sample, 300 μL of CSF (containing approximately 120 μg of protein) underwent protein precipitation using ice-cold ethanol. The resulting protein pellet was dissolved in a solution of 9 M urea (Bio-Rad, Hercules, CA), 2% 2-mercaptoethanol (J.T. Baker, Center Valley, PA), 2% IGEPAL (Sigma, St. Louis, MO), and 0.25% carrier ampholytes (Bio-Rad). The sample was then loaded directly into 18 cm, 3–10 nonlinear IPG strips (GE Healthcare, Little Chalfont, United Kingdom). A Protean IEF unit (Bio-Rad) was used to perform IEF at 20°C for a total of 100 kVh. The IPG strips were equilibrated in solutions containing DTT (Bio-Rad) and subsequently iodoacetamide (Fluka, St. Louis, MO) for reduction and alkylation of the focused proteins. Polyacrylamide gel electrophoresis was performed using 12–15% T gradient slab gels. The gels were fixed, stained with SYPRO Ruby Protein Gel Stain (Molecular Probes, Carlsbad, CA), and de-stained according to manufacturer's instructions. The gels were scanned on an FLA-3000 Fluorescent Image Analyzer (Fujifilm, Stamford, CT).

The resulting gel images were imported into the Melanie software package (version 4.0, GeneBio, Geneva, Switzerland). The software was used to auto-detect spots that were subsequently manually corrected to remove artifacts. Each sample gel was then matched to a

master gel image created by combining the spots present in four gels with the most number of spots. Matching was initially performed using the software's automated matching tool and then checked and corrected manually. The percent integrated intensity (% volume) of each matched spot was then exported. A preliminary assessment was conducted to identify spots that showed a consistent and considerable change in % volume between the CSF samples collected before IVIg therapy and after six months of therapy. Changes in spot % volume were arbitrarily deemed considerable if they were greater than two-fold in at least five of the subjects, and consistent if the same direction of change was observed in at least seven of the eight subjects.

2.3 Statistical analysis

A statistical analysis was carried out using JMP[®] 7.0 (SAS Institute Inc., Cary, NC). In this analysis, the % volume data from CSF samples at t=0 and t=6 (from all eight subjects) that showed a normal distribution, were fit to a linear mixed model using JMP Restricted Maximum Likelihood (REML) estimation method. In the Fit Model function of JMP, % volume data was chosen as a role variable. Time of CSF sample collection, IVIg dose, the cross interaction between time and dose were chosen as model effects. Dose was then nested within patients and was chosen as the random effect. This analysis identified 79 spots with a significant change in % volume after six months of IVIg therapy. The same statistical analysis was then applied to the % volume of these same 79 spots in samples collected after six months of IVIg therapy (t=6) and the washout period (t=9) from all eight subjects (except subject #5 for which a sample was not available). The purpose of this second analysis was to identify proteins, out of the pool of 79 spots, whose % volume changed significantly after IVIg therapy, with this change being significantly sustained or reversed during drug washout. The global risk was fixed at $p < 0.05$ for all tests.

2.4 Protein identification

Among the spots with a change in % volume with IVIg therapy, some protein identifications were made with a 2DE CSF map [19], others were made by using tryptic digestion followed by MS. The details of the digestion and MS protocols have been previously published [20]. The MS analysis was done using a 4800 MALDI TOF/TOF (AB Sciex, Framingham, MA). Peptide mass fingerprint data were collected in positive ion reflector mode in the range of 900 to 4000 mass to charge ratio. Several of the highest intensity non-trypsin peaks were then selected for tandem mass spectrometry analysis. MS/MS was collected in positive ion mode with default calibration. PMF and MS/MS spectra were analyzed using GPS Explorer (version 2.0, AB Sciex), which acts as an interface between the Oracle database containing raw spectra and a local copy of the Mascot search engine (version 2.0, Matrix Science, London, United Kingdom). Data were searched against a locally stored copy of the NCBI nr protein database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Protein&itool=toolbar>). A mass tolerance of 25 ppm was used for the PMF data and 0.2 Da for the MS/MS data. For a database match to be considered a positive identification, a value of $p < 0.05$ (calculated by GPS Explorer) was required in addition to at least one high confidence (95% confidence interval or higher) MSMS match.

3 Results

Typical CSF 2DE gels from samples from each timepoint of IVIg therapy are shown in Figure 1. The number of spots on 2DE gels did not change significantly over the course of the IVIg treatment. There were an average of 1178 $\pm$ 192 spots in the t=0 CSF gels and 1173 $\pm$ 161 spots in the t=6 CSF gels, as calculated from all gels from each timepoint. In an initial assessment, %volumes for 69 spots changed in a consistent and considerable manner between the t=0 and the t=6 CSF gels. The directions of change for these 69 2DE spots, along with information on the protein(s) identified in the spots, are summarized in Table 2. All of the 2DE spots in Table 2 (except for spots 6017 and 1378) decreased in %volume after IVIg treatment.

In the statistical analysis using JMP®, 79 spots were found to have undergone a significant change in %volume between the t=0 and the t=6 CSF gels. The analysis also showed that none of these spots changed with respect to dose or the interaction between time and dose. The second statistical analysis revealed that only 25 spots, out of the 79, changed in a way that was sustained or reversed in the t=9 CSF gels compared to the changes between t=0 and t=6 CSF gels. Table 3 lists the 25 proteins with their corresponding *p* values and the direction of change between t=0, t=6, and t=9 CSF gels.

Proteins in Table 3 can be categorized in two groups based on whether their concentration increased (green and black) or decreased (blue and red) after six months of therapy. Proteins within each of the above categories were further divided in two categories: those showing a sustained effect (black and blue) or those showing a reversion (green and red) during washout.

4 Discussion

Ideally, 2DE would result in a single protein per spot with each protein having a unique spot location based on molecular weight and isoelectric point. In practice, however, 2DE maps are complex because multiple proteins can migrate to a single spot and because proteins can undergo processing (e.g. post-translational modifications, cleavages, etc.), resulting in the possibility that a given protein may appear in many locations on a 2DE map. In our initial assessment, several proteins appeared at least four times in Table 2 but with different spot numbers, this is possibly due to the presence of isoforms, post-translational modifications, or degradation fragments. There are multiple spots of the same protein within Table 2, and between Tables 2 and 3, that exhibit different trends – for example transthyretin 4180 (Table 2) and transthyretin 4604 (Table 3). Such observations may result from changes in expression of particular forms of a protein. For example, others have reported differing expression levels for different AD CSF transthyretin protein spots on 2DE images [21]. Table 2 also contains proteins previously linked to AD such as apoE and apoJ [22–23].

Our analysis of the longitudinal changes in CSF protein expression using 2DE has resulted in some interesting observations. First, all spots with a known protein identity in Table 3 (some are also present in Table 2), have been previously implicated in AD. Second, the observed direction of changes for these proteins is consistent with previous reports regarding

the changes in their concentration in AD. Third, two of the proteins, Ig light chain and complement component 3, have also been previously proposed as diagnostic biomarkers of AD [22]. For discussion, the proteins in Table 3 are arranged in two categories: proteins with an increase in CSF expression after IVIg therapy, and proteins with a decrease in expression after therapy.

Ig, Ig light chain, kallikrein 6 preprotein, and transthyretin show elevated CSF expression after six months of IVIg therapy. In the case of the immunoglobulins, this increase is mitigated during washout which is consistent with the nature of immunotherapy and the transport of antibodies into CSF after peripheral administration. The increase in CSF expression of kallikrein 6 and transthyretin after therapy is consistent with previous reports regarding the reduced levels of these proteins in the CSF of patients with AD [24–26]. Kallikrein 6, a serine protease, is highly expressed in the brain and transthyretin has been shown to bind A β [24–25].

EPC-1, gelsolin, α 1-antitrypsin, albumin, and inter- α trypsin inhibitor all show a decrease in CSF expression after IVIg treatment, with a sustained effect during IVIg washout. The decrease in expression of EPC-1, gelsolin, and α 1-antitrypsin is consistent with previous studies showing increased expression of these proteins in the brain or CSF of AD patients [27–33]. Inter- α -trypsin inhibitor, a plasma protease inhibitor, has been shown to be related to the pathogenesis of AD, specifically by playing a role in the formation of senile plaques and neuronal degradation [34].

Fibrinogen γ , transferrin, complement factor H, and complement component 3 also show a decrease in CSF expression after IVIg treatment. However, this change is reversed after IVIg washout, which may imply that the effect of IVIg on these proteins is not as long term as on the proteins discussed above. The decrease in expression of fibrinogen, an A β binding protein, and transferrin is consistent with previous studies showing elevated levels of these proteins in the brain or CSF of AD patients [35–37]. With respect to the complement proteins, several studies have indicated that there is a change in complement activity in the brains of patients with AD [38], and that some of its isoforms bind A β . There were also 54 protein spots that demonstrated a change in expression that was continued even after IVIg administration had stopped. Such changes may reflect non IVIg-associated expression changes (perhaps due to aging over the time period studied) or changes induced by IVIg that carry over despite continued IVIg treatment.

This study is the first to report on the longitudinal analysis of CSF samples from a full set of clinical trial subjects to monitor therapy-induced changes in the CSF proteome over 9 months in discovery mode. It is also the first study to report on possible protein targets of IVIg in the CSF of AD patients although it is difficult to deconvolute the specific impact of anti-A β antibodies present in IVIg from the impact of IgGs in general. The results suggest that changes in the CSF proteome after IVIg therapy can be detected using 2DE when analyzed with robust statistical analyses and many of the changes identified in this study reinforce observations from a previous experiment in which CSF from two of the subjects were analyzed using a shotgun proteomics method [39]. At the same time, there are some differences between this study that analyzed all eight subjects and the previous study that

investigated only two subjects. Beside the important differences between shotgun and 2DE as analytical tools, the previous study identified 19 non-antibody proteins with increased expression and 7 with decreased expression in response to IVIG treatment; whereas, here we found only 3 proteins increased and 20 proteins decreased (15 with known identities) in response to IVIG treatment. Taken together and when combined with analyses of diagnostic biomarkers [40], a proteomic analysis of CSF may be useful in the search for biomarkers and possible surrogate endpoints to monitor a subject's response to therapy. Although additional studies with a larger cohort size are necessary to fully correlate the statistical results with clinical outcomes, something that may be possible as part of a phase III clinical trial, we believe that longitudinal AD CSF studies proposed in the context of therapy-induced changes are critically needed to identify diagnostic and/or treatment biomarkers of AD. Moreover, proteomics approaches to CSF analysis of longitudinal samples offers an important means to study biomarkers related to drug treatment and mechanism of action where individuals can serve as their own controls.

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Abbreviations

AD	Alzheimer's disease
CSF	cerebrospinal fluid
IVIg	intravenous immunoglobulin
Aβ	amyloid beta
MMSE	mini-mental status examination

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Figure 1.
Representative 2DE images of CSF from different time points. Proteins from Table 3 are labeled with their ID number.

Table 1^a Dosing and sample collection information for the eight AD subjects

Subject	IVIg dose (g/kg)	# weeks between doses	Time of lumbar puncture (weeks since IVIg started)	
			6 months after therapy	Washout period
1	1.0	2	23.85	36.85
2	0.4	1	25.14	35.71
3	0.4	2	22.85	34.42
4	2.0	4	27	40.71
5	1.0	2	26.42	--
6	0.4	1	27.14	41.28
7	0.4	2	27.14	41.28
8	2.0	4	27	41.42

^a Previously published in [40].

Table 2

2DE spots that show a consistent and considerable change in the % volume after six months of IVIg therapy

ID#	Protein ID	NCBI [30] Accession #	Observed/Calculated MW (kDa)	Direction of Change t=0 to t=6
2850	α 1-antitrypsin	1942629	52/44.3	-
2905	α 1-antitrypsin	1942629	51/44.3	-
2335	α 1-antitrypsin	1942629	48/44.3	-
5867	α 1-antitrypsin	1942629	18/44.3	-
6017	α 1-antitrypsin	1942629	16/44.3	+
1378	α -1-B-glycoprotein	69990	88/51.9	+
1010	Albumin	113576	105/69.4	-
1037	Albumin	113576	105/69.4	-
1593	Albumin	113576	72/69.4	-
1861	Albumin	113576	64/69.4	-
6031	Albumin	113576	53/69.4	-
2172	Albumin	113576	53/69.4	-
2185	Albumin	113576	51/69.4	-
5977	Albumin	113576	45/69.4	-
2863	Albumin	113576	38/69.4	-
3463	Albumin	113576	27/69.4	-
3458	Albumin	113576	27/69.4	-
4354	Albumin	113576	15/69.4	-
4537	Albumin	113576	14/69.4	-
3643	Albumin	113576	24/69.4	-
3481	Albumin	113576	28/69.4	-
2964	Apolipoprotein E	178853	37/36.2	-
3048	Apolipoprotein E	178853	35/36.2	-
5931	Apolipoprotein E	178853	15/36.2	-
2775	Apolipoprotein J	178855	40/48.8	-
2976	Apolipoprotein J	178855	34/48.8	-
1946	α -2-glycoprotein I	4557327	63/38.3	-
1432	BiP Protein	6470150	88/70.9	-
1804	Complement factor B	291922	65/85.5	-
1133	Contactin 1	28373119	100/112	-
712	Contactin 2	4827022	128/113	-
713	Contactin 2	4827022	128/113	-
3204	Cyclin D-1	483601	32/7.6	-
3065	EPC-1	1144299	33/40.1	-
3260	EPC-1	1144299	31/40.1	-
2179	EPC-1/Albumin	182424	75/69.8	-
1566	Fibrinogen alpha	4504165	100/85.7	-
1168	Gelsolin	4504165	100/85.7	-
1174	Gelsolin	4504165	100/85.7	-

ID#	Protein ID	NCBI [30] Accession #	Observed/Calculated MW (kDa)	Direction of Change t=0 to t=6
1169	Gelsolin	4504165	100/85.7	-
1177	Gelsolin	4504165	100/85.7	-
4682	Gelsolin	4504165	80/85.7	-
1440	Gelsolin	4504165	65/85.7	-
1839	Gelsolin	229908	36/25.2	-
2989	Malate dehydrogenase	14585855	24/25.7	-
903	Plasminogen	135807	95/70.0	-
1272	Prothrombin	553788	80/53.8	-
1368	Transferrin	553788	80/53.8	-
1393	Transferrin	553788	44/53.8	-
2513	Transferrin	339685	19/12.8	-
4180	Transthyretin	72146	13/54.3	-
4648	Vitronectin	--	120/--	-
858	Unknown	--	105/--	-
1054	Unknown	--	100/--	-
1231	Unknown	--	80/--	-
1523	Unknown	--	75/--	-
5958	Unknown	--	70/--	-
1559	Unknown	--	65/--	-
1796	Unknown	--	58/--	-
2051	Unknown	--	53/--	-
2545	Unknown	--	41/--	-
2694	Unknown	--	35/--	-
3009	Unknown	--	33/--	-
3249	Unknown	--	27/--	-
3464	Unknown	--	24/--	-
3653	Unknown	--	23/--	-
5919	Unknown	--	23/--	-
3784	Unknown	--	19/--	-
4307	Unknown	--	18/--	-

2DE spots that show a statistically significant change in % volume after six months of IVIg therapy followed by a sustained or reversed effect during washout

Table 3

ID#	Protein ID	NCBI [30] Accession #	MW (kDa)	Direction of Change	p Value
3384 *	Kallikrein 6 preprotein	4506155	27/26.9	0<6=9	0.0064
5863	Unknown	--	25/--	0<6=9	0.0198
4604	Transthyretin	339685	14/12.8	0<6=9	0.0396
1048	Ig	226787	95/25	0<6>9	0.0089
3548	Ig light chain	21669471	25/28.2	0<6>9	0.0098
2193	Fibrinogen γ	223170	53/46.2	0>6<9	0.0017
1869	Albumin	113576	63/69.4	0>6<9	0.0028
2271 *	Complement factor H	758073	70/51	0>6<9	0.0029
3260	Unknown	--	30/--	0>6<9	0.0033
1488	Complement component 3	4557385	78/187	0>6<9	0.007
1762	Unknown	--	45/--	0>6<9	0.0076
1368	Transferrin	553788	85/53.8	0>6<9	0.0094
3204	Unknown	--	32/--	0>6<9	0.0167
1768	Unknown	--	68/--	0>6<9	0.0174
2179	EPC-1	1144299	53/40.1	0>6=9	0.0004
1169 *	Gelsolin	4504165	100/85.7	0>6=9	0.0005
2850	α 1-antitrypsin	1942629	52/44.3	0>6=9	0.0005
1168	Gelsolin	4504165	100/85.7	0>6=9	0.0013
3232	Albumin	113576	32/69.4	0>6=9	0.0032
858 *	Inter- α trypsin inhibitor	Q14624	120/103.3	0>6=9	0.0045
1177	Gelsolin	4504165	100/85.7	0>6=9	0.0068
1181	Gelsolin	4504165	100/85.7	0>6=9	0.0068
2858	Albumin	113576	40/69.4	0>6=9	0.0073
3435	Unknown	--	28/--	0>6=9	0.0084
1975	Albumin	113576	65/69.4	0>6=9	0.015

* identified in this study

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Color key:

	% volume after 6mo			
	increased	decreased		
	black	blue	sustained	reversed
trend after washout				
	green	red		