Journal of Alzheimer's Disease 47 (2015) 873–876
DOI 10.3233/JAD-150382
IOS Press

Short Communication

Cerebrospinal Fluid from Alzheimer's Disease Patients Contains Fungal Proteins and DNA

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Accepted 11 May 2015

Abstract. The identification of biomarkers for Alzheimer's disease is important for patient management and to assess the effectiveness of clinical intervention. Cerebrospinal fluid (CSF) biomarkers constitute a powerful tool for diagnosis and monitoring disease progression. We have analyzed the presence of fungal proteins and DNA in CSF from AD patients. Our findings reveal that fungal proteins can be detected in CSF with different anti-fungal antibodies using a slot-blot assay. Additionally, amplification of fungal DNA by PCR followed by sequencing distinguished several fungal species. The possibility that these fungal macromolecules could represent AD biomarkers is discussed.

Keywords: Cerebrospinal fluid biomarker, fungal DNA, fungal infection, neurodegenerative disease, protein biomarker

The possibility that Alzheimer's disease (AD) is caused by a microbial infection has been considered by a number of laboratories [1–4]. Several animal viruses or bacteria have been suggested as the potential etiology or as a risk factor for AD. For example, herpes simplex virus type 1 (HSV-1) has been related to AD [5–7], and viral genomes can be detected in amyloid plaques [8]. However, no significant differences were found between normal elderly people and AD patients for the presence of herpes DNA and signs of viral replication [9]. Additionally, DNA from *Chlamidophyla pneumoniae* (formerly known as *Chlamydia pneumoniae*) has been detected in a large percentage of AD brains [10]; however, these findings could not be reproduced by other researchers [11, 12]. Antigens and DNA from several infectious agents have been found in cerebrospinal fluid (CSF) from AD sufferers [1, 3, 4], including HSV-1 and *C. pneumoniae* [13, 14]. More recently, we have proposed that disseminated mycoses may play a part in the etiology of AD. Accordingly, increased amounts of fungal polysaccharides, proteins, and DNA were observed in peripheral blood from AD patients [15] and proteomic and gene sequencing analysis revealed proteins in AD brains and also DNA from several species of fungi [16]. Moreover, yeast cells can be directly visualized in AD brains, both intra- and extracellularly, by immunohistochemistry using specific polyclonal antibodies raised against fungi [17].

In the present work, we have evaluated whether fungal proteins and DNA can be detected in CSF from AD patients. As a first test, we used a slot-blot protocol as

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ISSN 1387-2877/15/$35.00

Table 1
Fungal antigens present in CSF samples from AD patients

Patients	Age	Gender	*C. famata*	*C. albicans*	*C. glabrata*	Enolase	β tubulin peptide
Patient 1	91	Female	154 ± 6.2	143 ± 5.7	104 ± 5.2	173 ± 8.6	114 ± 5.7
Patient 2	80	Male	ND	65 ± 3.2	ND	157 ± 4.5	30 ± 2.4
Patient 3	98	Female	77 ± 3.8	77 ± 3.8	53 ± 4.2	132 ± 4.3	225 ± 11.2
Patient 4	74	Female	9 ± 0.6	57 ± 3.4	37 ± 2.9	109 ± 3.9	47 ± 3.8
Patient 5	93	Female	1 ± 0.1	56 ± 3.5	44 ± 3.5	84 ± 3.6	78 ± 3.8
Patient 6	77	Female	26 ± 2.2	63 ± 3.2	22 ± 1.8	145 ± 5.8	82 ± 4.1
Patient 7	86	Male	16 ± 1.3	10 ± 1.2	29 ± 2.3	14 ± 1.2	1 ± 0.1
Patient 8	82	Male	42 ± 3.4	ND	26 ± 2.1	107 ± 5.3	7 ± 0.6
Patient 9	88	Female	74 ± 4.0	75 ± 4.1	37 ± 3.0	142 ± 7.1	34 ± 2.7
Patient 10	85	Male	122 ± 4.9	77 ± 3.7	130 ± 6.5	154 ± 7.7	63 ± 3.1
Mean value			57.8	69.2	53.5	121.7	68.1
Control 1	64	Male	26 ± 2.1	13 ± 0.9	15 ± 1.2	79 ± 3.9	10 ± 0.7
Control 2	43	Male	9 ± 0.7	20 ± 1.5	88 ± 2.8	79 ± 3.9	81 ± 3.2
Control 3	68	Female	1 ± 0.1	1 ± 0.1	5 ± 0.4	ND	1 ± 0.2
Mean value			12.0	11.3	36.0	79.0	30.6
P value			0.31	0.05	0.04	0.04	0.12
Odds ratio			4.0	16.0	18.0	18.0	8.0

The mean of the densitometric values of three experiments and the standard deviation are indicated. ND, not done.

previously described to measure fungal antigens [15]. Briefly, triplicate 40 μl samples of CSF were filtered onto nitrocellulose membranes and incubated with a range of polyclonal rabbit antibodies (1:500 dilution) raised against different species of fungi [18–20]. Following incubation with a horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:5000 dilution, Amersham Biosciences), the membrane was exposed to X-ray film and densitometric analysis was performed. Quantification obtained with each anti-fungal antibody in CSF samples from AD patients and controls is shown in Table 1. The majority of CSF samples from AD patients gave high densitometric values with at least one of the antibodies, which were above the cut-off values reported previously [18, 19]. This was the case for AD patients 1–6, 9, and 10, whereas CSF from patients 7 and 8 produced rather low values with the antibodies tested. The *p* value and the odds ratio for each antibody employed are shown in Table 1. Considering all the results generated, a global p value of 0.0016 and an odds ratio of 8 were obtained. Statistical analyses of the data obtained were carried out using the Stata Program Version 11.0. One possible explanation for the evident low values in patients 7 and 8 is that they do not have a mycosis, or perhaps the infection is present in the CNS but not the CSF. Alternatively, the antigens present in these patients may belong to a species of fungus that does not crossreact with the antibodies employed in this study. Yet another possibility is that the levels of fungal antigens in CSF fluctuate during the course of the disease. Elevated levels of these antigens in CSF have been previously reported by our laboratory in patients with multiple sclerosis or amyotrophic lateral sclerosis [18, 19], pointing to the concept that mycoses also exists in these two neurodegenerative diseases. These disseminated fungal infections may contribute as a risk factor for these diseases or can play a part in their etiology. Thus, in several neurodegenerative diseases so far analyzed, CSF from some patients contains high levels of fungal antigens.

A second sensitive test for mycosis detection is the analysis of fungal DNA sequences after PCR amplification. We have developed several nested PCR-based

Table 2
Fungal species present in CSF samples from AD patients detected by PCR

Species	Patient 1	Patient 2	Patient 4	Patient 5	Patient 7	Patient 9
Candida albicans	2;3			2	1	1
Cladosporium				3		
Cryptoccocus	1		1;2	1		2
Malasezzia globosa		1	1	1		
Malasezzia restricta	1					1
Sacharomyces cerevisae						3

Numbers refer to the PCR amplification schedules that were positive for a given species. PCR 1: External ITS1 + ITS-1 (internal 1). PCR 2: External ITS1 + ITS-1 (internal 2). PCR 3: External ITS1 + ITS-1 (internal 3).

assays to amplify the internal transcribed spacer 1 (ITS-1) of the fungal genome [16, 18]. Amplified products can be separated on agarose gels and sequencing of the corresponding PCR fragments establishes the species present. The specific oligonucleotide primers and conditions employed in these PCR assays have been described in detail [18]. Special care should be taken to avoid contamination during the PCR protocol and the DNA extraction method. Using this procedure, DNA was extracted from 200 μl of CSF using the QIAmp (Qiagen) Genomic DNA Isolation Kit. Sufficient material was only available from six patients as indicated in Table 1. After DNA extraction, three PCR assays were carried out. After a first-round PCR of the ITS-1 region with external primers, three further rounds of PCR were performed using different internal primers (see scheme Supplementary Figure 1). A typical PCR result is shown in Supplementary Figure 1. As can been seen in this Figure, no DNA products were amplified from controls for the PCR assay and for the DNA extraction method. Also, no DNA fragments appeared from DNA extracted from control CSFs, suggesting that there is no skin contamination. The products obtained with the three PCR were separated on agarose gels and each individual band was sequenced. All six CSF samples contained fungal DNA and the species identified in each sample are shown in Table 2. The species detected are potential human pathogens and some of them have been previously found in brain samples from AD patients [16]. Curiously, only two out of six CSF samples contained one fungal species, whereas in the other four patients mixed fungal infections were revealed. Of interest, AD patient 7, who was negative for fungal protein in the slot-blot assay, was positive for fungal DNA. Collectively, these data demonstrate that DNA from several fungi can be detected in CSF from AD patients and, more importantly, that the species can be identified using this technique.

The search of potential biomarkers for neurodegenerative diseases in CSF has identified a number of proteins that are altered in these patients [21–25]. This is the case for amyloid-β peptide, which is present in reduced amounts in CSF from AD patients, whereas tau protein is increased [26–28]. Interestingly, similar alterations in these proteins are also observed in patients with CNS infections, including viral, bacterial, protozoal and fungal infections [13, 29]; however, the concentration of phosphorylated tau is usually normal in some of these infections. These parallels between the altered levels of amyloid and tau proteins in some neurodegenerative diseases and microbial infections have been ascribed to the induction of neuroinflammation. Alterations in other proteins have also been proposed as potential biomarkers for these diseases, for example, the elevation in chitinase levels in [30–34] CSF from AD, multiple sclerosis, and amyotrophic lateral sclerosis patients. We have recently suggested that this increase in chitinase may be related to the presence of its substrate, i.e., fungal chitin [18]. In this regard, our present findings provide additional support for the possibility that components from different fungi are present in AD patients. The anti-sera employed in this work cross-react with a number of fungal proteins of species different from the one used to obtain the antiserum. Therefore, the evidence of high levels of fungal proteins in CSF using a given antiserum does not mean that the fungal proteins present in CSF belong to the antiserum species. Thus, the existence of fungal proteins in CSF is indicative of a disseminated mycosis, but does not provide unequivocally the fungal species present. This can be achieved by DNA sequencing. Thus, fungal macromolecules in CSF might constitute new biomarkers for AD. Moreover, proteins from fungi can be identified in peripheral blood serum, representing a much less invasive technique for patients [15]. The presence of fungal DNA is potentially more interesting because it can indicate not only that a mycosis exists, but also serves to identify the species responsible for the disseminated infection. In light our findings, it will be interesting in future studies to analyze fungal proteins and DNA in CSF from patients with early cognitive decline, or even before the onset of AD symptoms are apparent. These analyses will indicate to what extent the presence of fungal macromolecules in CSF can be of prognostic value for AD.

ACKNOWLEDGMENTS

The financial support of Fundación ONCE (Organización Nacional de Ciegos Españoles) is acknowledged. We acknowledge an institutional grant to Centro de Biología Molecular “Severo Ochoa” from the Fundación Ramón Areces.

Authors’ disclosures available online (http://j-alz.com/manuscript-disclosures/15-0382).

SUPPLEMENTARY MATERIAL

The supplementary table and figure are available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-150382.

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