I. ABSTRACT

Dementia in Alzheimer’s disease (AD) is ultimately due to cell loss mediated by several mechanisms including, apoptosis, impaired mitochondrial function, and possibly necrosis. A second major neuroanatomic correlate of dementia is aberrant cortical neuritic sprouting with abundant proliferation of dystrophic neurites. Early in vivo detection of AD will require non-invasive assays of highly sensitive and relatively specific biomarkers that reflect these fundamental abnormalities in cellular function. The AD-associated neuronal thread protein (AD7c-NTP) gene encodes a ~41 kD membrane-spanning phosphoprotein that causes apoptosis and neuritic sprouting in transfected neuronal cells. The AD7c-NTP gene is over-expressed in AD beginning early in the course of disease. In the brain, increased AD7c-NTP immunoreactivity is associated with phospho-tau-immunoreactive cytoskeletal lesions, but not with amyloid-β accumulations. The levels of AD7c-NTP in postmortem brain tissue correlate with the levels measured in paired ventricular fluid samples, suggesting that the protein is secreted or released by dying cells into cerebrospinal fluid (CSF). In this regard, elevated levels of AD7c-NTP can be detected in both CSF and urine of patients with early or moderately severe AD, and the CSF and urinary levels of AD7c-NTP correlate with the severity of dementia. The newest configuration of the AD7c-NTP assay, termed “7c Gold”, has greater than 90% sensitivity and specificity for detecting early AD. The aggregate results from a number of studies suggest that AD7c-NTP is an excellent biomarker that could be helpful in the routine clinical evaluation of elderly patients at risk for AD.

2. RATIONALE FOR CONSIDERING NOVEL BIOMARKERS FOR THE DETECTION AND DIAGNOSIS OF ALZHEIMER’S DISEASE

Dementia in Alzheimer’s disease (AD) is ultimately mediated by cell loss which studies have demonstrated occurs by several mechanisms including, apoptosis (1, 13, 19, 32, 55), impaired mitochondrial function (4, 11), and possibly necrosis (18, 43, 52, 54). Apoptosis is detectable in both neuronal and non-neuronal cells, indicating that multiple cell types are adversely affected by neurodegeneration. A second major correlate of dementia in AD is aberrant cortical neuritic sprouting with abundant proliferation of dystrophic neurites that are immunoreactive for phosphorylated neuronal cytoskeletal proteins, as well as other proteins such as growth-associated synaptic molecules and nitric oxide synthase-3.
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(7, 12, 16, 27, 37, 38, 51). Whether the neuritic pathology represents a primary degenerative lesion, a secondary response to cell loss, or a combination of both, remains to be determined.

Within the last decade, extensive research aimed at determining the mechanisms of neuronal cell death has revealed important differences between aging and neurodegeneration of the CNS, with one of the critical distinctions being that brains with AD exhibit heightened sensitivity to oxidative stress and free-radical injury. Although genetic factors such as mutations in the presenilin (33, 49) or amyloid precursor protein gene (47, 57), and the homozygous Apolipoprotein E4 genotype (3, 5, 26, 44) may predispose individuals to AD, sporadic (non-genetic) AD, which accounts for the vast majority of cases, is often associated with dysregulated expression genes that may render the brain cells more susceptible to oxidative injury. For example, aberrant expression of genes that encode superoxide dismutase I (35), nitric oxide synthase 3 (7, 10), or hemeoxygenase-1 (36, 46, 56), could result in increased generation of free-radicals, reduced CNS cellular resistance to oxidative stress and cytotoxic agents, and constitutive activation of pro-apoptosis mechanisms. In addition to abnormalities in gene expression, the progression of AD has been linked to chronic disturbances in CNS function caused by impaired energy metabolism (31), cerebral hypoperfusion (17), amyloid-β toxicity (45, 50), accumulation of non-amyloid-β precursor protein (α-synuclein) (28, 29), impaired calcium homeostasis (20), activation of pro-inflammatory cytokines (39), and reduced mitochondrial function (4, 11, 31). The aggregate findings in this body of work support a “multiple-hit” hypothesis proposing that several abnormalities in cell biology and function contribute to AD neurodegeneration. Therefore the study of AD should be approached by examining early alterations in gene expression that adversely affect various aspects of neuronal function.

3AD7c-NTP: MOLECULAR CHARACTERIZATION AND POTENTIAL RELEVANCE TO NEURODEGENERATION

3.1. Overview

Investigations focused on identifying early gene abnormalities in AD led to the discovery of the AD7c-NTP (NTP = neuronal thread protein) gene (9). AD7c-NTP is a novel cDNA that encodes a ~41 kD membrane-spanning phosphoprotein with putative domains that may account for some of the cellular responses associated with its over-expression in AD and in transfected neuronal cells. In this review, we summarize evidence that: 1) the AD7c-NTP gene is over-expressed in brains with AD; 2) the corresponding ~41 kD protein accumulates in neurons with progression of AD; 3) AD7c-NTP protein co-localizes with neurodegenerative lesions that correlate with dementia; 4) over-expression of AD7c-NTP early in the course of disease is detectable in cerebrospinal fluid and urine; and 5) over-expression of AD7c-NTP in transfected neuronal cells causes apoptosis, impaired mitochondrial function, and increased neuritic sprouting. Importantly, the AD7c-NTP gene is aberrantly expressed in sporadic AD, which accounts for the vast majority of the cases.

3.2. Characteristics of the AD7c-NTP gene (9)

The AD7c-NTP cDNA was isolated from a library prepared with temporal lobe mRNA from a brain with definite AD. The library was screened using polyclonal antibodies to human pancreatic thread protein, which cross-react with an epitope on NTP, despite the fact that the two proteins are clearly distinct. The 1442 bp AD7c-NTP cDNA contains 1125-nucleotides of open reading frame. Bestfit and GAP analyses revealed four Alu-type sequences embedded in the open reading frame, and a near-duplication (85% identical) of the first 450 nucleotides starting at nucleotide 898. The translated 375 amino acid sequence has a predicted molecular weight of 41,718 and, Kyte-Doolittle and Chou-Fasman hydrophilicity and Hopp-Woods surface probability profiles predict a 15 amino acid hydrophobic leader sequence, and at least one membrane-spanning region. Subsequence analysis demonstrated 17 cAMP, calmodulin-dependent protein kinase II, protein kinase C, or glycogen synthase kinase 3 phosphorylation sites, and one myristoylation site. In addition, a single TGFβ motif was detected. The translated molecule contains a region that is 75% identical to a region of the IGF1/insulin receptor hybrid protein. These features suggest that the AD7c-NTP protein is a membrane-bound or membrane-associated phosphoprotein that could be secreted, and possibly modulated by IGF1 or insulin stimulation.

3.3. Effects of AD7c-NTP over-expression in cultured neuronal cells

Transfection of human neuronal cells with the AD7c-NTP cDNA to over-express the gene has two effects: 1) it causes cell death; and 2) the remaining viable cells exhibit prominent neuritic sprouting (9). In contrast, over-expression of AD7c-NTP has no effect on DNA synthesis. More recently, we demonstrated that over-expression of the AD7c-NTP gene causes neuronal cell death mediated by apoptosis and impaired mitochondrial function, with activation of the pro-apoptosis pathways observed in brains with AD (14). In addition, the remaining cells exhibit prominent neuritic sprouting with increased expression of synaptophysin, phospho-tau, but not amyloid-β (14). It is noteworthy that in AD, apoptosis (1, 6, 19, 32, 40, 53-55) and impaired mitochondrial function (4, 11) are important mechanisms of cell loss, and that aberrant cortical neuritic sprouting is a significant correlate of dementia (12, 27, 37, 38, 51). Further investigations revealed that primary CNS neuronal cells transfected with the AD7c-NTP cDNA also exhibit reduced viability followed by prominent neuritic sprouting in residual viable neurons (15). Current investigations are focused on further characterizing the mechanisms of AD7c-NTP-induced neuronal apoptosis and neuritic sprouting. Our hypothesis is that over-expression of the AD7c-NTP gene leads to constitutive activation of other genes such as nitric oxide synthase-3, which in turn increase cellular sensitivity to oxidative stress and free radical injury. We are also examining the hypothesis that secreted AD7c-NTP protein may promote either a pro-apoptotic or sprouting response in “target” neurons.

4. ANALYSIS OF AD7c-NTP GENE EXPRESSION IN RELATION TO THE PATHOLOGY OF ALZHEIMER’S DISEASE

4.1. Increased levels of AD7c-NTP mRNA expression in brains with AD

To demonstrate expression of the AD7c-NTP gene in the brain, it was necessary to detect the corresponding mRNA transcripts. Therefore, AD7c-NTP mRNA expression was examined in frontal (Brodmann
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Area 11) and temporal (Area 21) lobe postmortem tissue by Northern blot analysis and In situ hybridization (9). Radiolabeled AD7c-NTP cDNA probes detected the expected 1.4 kB mRNA as well as a highly related 0.9 kB transcript in adult human brains, but not in other tissues including pancreas, intestines, liver, lung, ovary, and testis. A series of AD and aged control brain samples were analyzed by Northern blot analysis, and the levels of gene expression were quantified by densitometry.1—3 Statistical analysis of those results revealed significantly higher mean levels of both the 1.4 kB (P<0.005) and 0.9 kB (P<0.05) AD7c-NTP transcripts in the AD relative to the normal aged control brains.

In situ hybridization was performed with antisense and sense (negative control) AD7c-NTP cRNA probes labeled with (11-digoxigenin)UTP (2). Specifically bound probe was detected with alkaline phosphatase-conjugated anti-digoxigenin and the BCIP/NBT substrate. AD7c-NTP-related mRNA transcripts were detected in cortical neurons in both AD and aged control brains, but higher levels of expression were observed in AD, corresponding with the results of Northern blot analysis. In addition, low levels of AD7c-NTP mRNA transcripts were detected in cortical and white matter glial cells in the brains with AD. Therefore, the results obtained by Northern blot analysis and In situ hybridization indicate that AD neurodegeneration is associated with constitutively increased levels of AD7c-NTP mRNA expression in the brain.

4.2. Elevated levels of AD7c-NTP protein with AD neurodegeneration

To examine AD7c-NTP immunoreactivity, we generated polyclonal and monoclonal antibodies (mAbs) to the recombinant protein (8). Binding specificity for each antibody was demonstrated by Western blot analysis and sandwich immunoassays. Using the N3H4 mAb generated to AD7c-NTP, we detected expression of the expected ~41 kD protein, as well as an ~18-21 kD AD7c-NTP-related protein in both AD and aged control brains by Western blot analysis. The ~18-21 kD AD7c-NTP-related protein may represent cleavage products of the ~41 kD molecule, or a distinct protein encoded by the 0.9 kB transcript. The levels of AD7c-NTP proteins were quantified by densitometric analysis of non-saturated autoradiographs. Statistical analysis of data demonstrated significantly higher levels of both the ~41 kD and the 18-21 kD AD7c-NTP-immunoreactive proteins in AD relative to aged control frontal and temporal lobe samples (t=9.5; 19 df; P<0.0001). Further comparisons made between early and late (end-stage) AD revealed generally higher levels of both AD7c-NTP-immunoreactive proteins in brains with end-stage disease, suggesting that these molecules accumulate in the brain with progression of AD.

In addition to Western blot analysis, we used a highly sensitive enzyme-linked sandwich immunosorbant assay (ELSIA) to measure AD7c-NTP protein levels in brain tissue homogenates. The ELSIA utilizes polyclonal anti-AD7c-NTP bound to a solid phase support to capture AD7c-NTP proteins, and non-isotopically labeled high affinity N3H4 or N2C11 monoclonal antibody to detect the captured molecules. Capillary electrophoresis studies demonstrated a single ~41 kD peak, indicating that the ELSIA selectively detected the ~41 kD AD7c-NTP, and not the ~18-21 kD AD7c-NTP-related protein. The AD7c-NTP ELSIA can measure as little as 10 picomoles of specific protein with less than 10% coefficient of variation. Using the ELSIA to analyze a large number of samples, we demonstrated significantly higher mean levels of AD7c-NTP protein in AD relative to age-matched control postmortem temporal lobe homogenates (8, 9), thus confirming the results obtained by Western blot analysis and indicating that AD7c-NTP protein accumulates in brains with AD.

Subsequent analysis of the translated cDNA suggests that AD7c-NTP is a phosphoprotein. In this regard, we have obtained preliminary evidence that the AD7c-NTP protein can be phosphorylated by demonstrating phospho-serine and phospho-threonine immunoreactivity in AD7c-NTP immunoprecipitates from human PNET2 neuronal cells transfected with the AD7c-NTP cDNA (unpublished observation). Further studies to formally prove that AD7c-NTP can be phosphorylated by specific kinases are currently in progress.

Since phosphorylation of a protein can dictate its biological effects, we conducted studies to determine if the AD7c-NTP protein was phosphorylated in vivo. To do this, proteins were extracted from cerebral tissue in buffer supplemented with phosphatase inhibitors. Western blot analysis detected ~41-45 kD rather than ~41 kD AD7c-NTP-immunoreactive proteins, and elevated levels of these additional forms of AD7c-NTP in brains with late-stage compared with early-stage AD (8, 9). Presumably, the variably retarded migration of AD7c-NTP-immunoreactive molecules was due to different degrees of phosphorylation. If this interpretation is correct, the results would suggest that the phosphorylation state of AD7c-NTP protein accumulated in brains with AD increases with progression of disease.

4.3. Profiles of AD7c-NTP immunoreactivity in the brain

Neurofibrillary tangles, dystrophic neurites, senile plaques, and amyloid-β (Aβ) peptide deposits in the brain represent characteristic neuropathologic abnormalities in AD. The abundant localization of these lesions in corticolimbic structures may account for some of the early clinical manifestations of the disease, including cognitive impairment, memory loss, personality change, and behavioral disturbances. One of our interests was to determine the potential relationship between aberrant AD7c-NTP expression and these typical AD lesions. To do this, paraffin sections of Brodmann Areas 11 (frontal) and 21 (temporal) from AD and control brains were immunostained with a battery of monoclonal antibodies to AD7c-NTP, and adjacent sections were double-labeled to co-localize AD7c-NTP with phospho-tau-, phospho-neurofilament, AβB, or Aβ-immunoreactive lesions. The sections were immunostained using the avidin-biotin
horseradish peroxidase complex or the avidin-biotin alkaline phosphatase method (8).

AD7c-NTP immunoreactivity was localized in neuronal perikarya, neuropil fibers, and white matter fibers. The AD brains exhibited increased intensity of AD7c-NTP immunoreactivity in neuronal perikarya, and higher percentages of labeled cells within the cerebral cortex. Of note was that increased AD7c-NTP immunoreactivity was most prominent in neurons that appeared to be either cytologically intact or slightly degenerated due to their irregular cellular contours, faded nuclear membrane, and cell body shrinkage, with or without intracellular neurofibrillary tangles. In contrast, apoptotic cells and extracellular neurofibrillary tangles were not immunoreactive with the AD7c-NTP antibodies.

The battery of monoclonal antibodies generated to recombinant AD7c-NTP protein exhibited differential binding patterns in tissue sections, although all specifically recognized the ~41 kD recombinant protein, and the endogenously expressed ~41-45 kD brain proteins by Western blot analysis. Detailed binding studies demonstrated that the antibodies selected for study recognize different regions of the AD7c-NTP molecule. Therefore, the different patterns of labeling observed by immunohistochemical staining could reflect differences in protein conformation and epitope exposure. Four AD7c-NTP mAbs (N2U6, N3C11, N2S6, N2T8) exhibited intense degrees of immunoreactivity in AD cortical neurons, particularly pyramidal cells in layers 3 and 5. Two mAbs (N2U6, N2S6) prominently labeled neuropil and white matter fibers (axons), and 5 (N2U6, N3C11, N2S6, N2T8, N2J1) detected AβB+ and GFAP+ protoplasmic (Type 2) astrocytes in the cerebral cortex and white matter. Two AD7c-NTP mAbs (N2-U6 and N2-T8) also exhibited intense labeling of cortical neurons and swollen, irregular (dystrophic) neuropil neurites in AD, but low-level or absent labeling in aged control brains. Most striking was the immunoreactivity observed in AD-associated neurodegenerative lesions using the N2S6, N2T8, N3D12, and N2U6 mAbs, N2T8 detected intracellular neurofibrillary tangles, as well as degenerated neurons without neurofibrillary tangles; N3D12, N2T8, and N2J1 labeled swollen axons, dystrophic neurites, and fine wavy irregular thread-like structures (axons), particularly in superficial layers of the cerebral cortex of AD brains. The negative control 5C3 mAb to Hepatitis B (59) was not immunoreactive with adjacent sections of brain. The finding that several monoclonal antibodies were immunoreactive with AD and not normal brains suggests that the conformation of AD7c-NTP protein accumulated in AD brains may differ from that present in normal brains, perhaps due to increased phosphorylation.

To determine the relationship between AD7c-NTP immunoreactivity and AD lesions, we performed double-labeling studies to co-localize AD7c-NTP with phospho-tau, phospho-neurofilament, AβB+, or Aββ (8). As expected, the antibodies to phospho-neurofilament and phospho-tau detected neurofibrillary tangles, dystrophic neurites, neuritic plaques, and swollen irregular axons in AD brains. Corresponding with previous reports (8, 21, 58), AβB+ immunoreactivity was also strikingly increased in AD brains, and localized to neurofibrillary tangles, dystrophic neurites and glial cells identified as protoplasmic Type 2 astrocytes (GFAP+) and oligodendrocytes (galactocerebroside+) (13). Anti-AβB+ recognizes a membrane ganglioside expressed in some neurons, injured or immature oligodendrocytes, and protoplasmic (Type 2) astrocytes of a distinct cellular lineage (41, 42, 48). The anti-Aββ detected abundant Aβ deposits in senile plaques, dystrophic neurites, and blood vessels in the AD cases. In the double-labeling studies, AD7c-NTP was detected by the ABC method using DAB as the chromogen, while binding of the second primary antibody was detected with avidin-biotin-alkaline phosphatase reagents and the BCIP/NBT chromogen.

The double-labeling studies revealed AD7c-NTP immunoreactivity co-localized with phospho-tau, phosphorylated high- and middle-molecular weight neurofilament, and AβB+ in neuronal perikarya, abnormal neurites, and swollen axons. However the patterns of double-labeled cells were non-uniform, and many cells and cell processes exhibited immunoreactivity for only AD7c-NTP or phospho-tau, phospho-neurofilament, or AβB+. With regard to cells that were double-labeled, most histologically intact or slightly degenerated neurons primarily exhibited increased AD7c-NTP immunoreactivity with no perikaryal accumulations of phospho-tau, phospho-neurofilament, or AβB+, whereas neurons with more striking degrees of cytological degeneration manifested variable levels of both AD7c-NTP and phospho-tau (or neurofilament) immunoreactivity co-localized in the perikarya. The most conspicuous cytological degeneration, including the presence of well-defined neurofibrillary tangles, was associated with predominantly phospho-tau, and low-level or absent AD7c-NTP expression. In contrast, ghost (apoptotic) neurons were not labeled with any of the antibodies used. These findings suggest that there may be sequential but overlapping waves of altered immunoreactivity such that increased expression of AD7c-NTP is detectable early and in histologically normal-appearing neurons. With increasing neuronal degeneration, both AD7c-NTP and phospho-tau immunoreactivity increase, but as neurofibrillary tangles develop, AD7c-NTP becomes virtually undetectable, whereas phospho-tau, phospho-neurofilament, and AβB+ immunoreactivity abound.

With regard to the dystrophic neurites and swollen irregular axons, although many were double-labeled, a nearly equal proportion displayed immunoreactivity for only AD7c-NTP or phospho-tau, phospho-neurofilament, or AβB+. Moreover, unlike the profiles observed for neuronal labeling, there was no apparent progression in the pattern of neuritic labeling. This difference from the findings in neuronal perikarya could be explained by the fact that many of the neuritic processes detected in the cerebral cortex are likely to have originated from different and distant brain regions that exhibit variable degrees of AD neurodegeneration.

Aberrant AD7c-NTP was also detected in glial cells, many of which were AβB+. Most of the AβB+ glial
cells were characterized as protoplasmic (Type 2) astrocytes, based on the co-immunolocalization of GFAP. However, oligodendrocytes were also labeled, as judged by nuclear morphology and immunoreactivity for galactocerebroside. In AD, $A_{3}B_{5}$ was associated with amyloid-beta (13, 21, 34, 58). The aberrant AD7c-NTP expression in relation to glial cell degeneration (13, 21, 34, 58).

Finally, we sought to determine if the aberrant expression of AD7c-NTP was associated with amyloid-beta ($A_{3}$) deposits in the brain. The double-labeling studies performed as described above, revealed dual participation of $A_{3}$ and AD7c-NTP. Type 2 astrocytes were abundantly distributed in the cerebral cortex and white matter, but especially within the distribution of sub-cortical U-fibers. With the growing evidence that the cellular pathology in AD is not confined to neurons, these results suggest a potential role for AD7c-NTP expression in relation to glial cell degeneration (13, 21, 34, 58).

5. AD7c-NTP AS A BIOMARKER AND DIAGNOSTIC AID FOR ALZHEIMER’S DISEASE

5.1. Detection of AD7c-NTP in Postmortem Ventricular Fluid

Given the presence of a hydrophobic leader sequence and myristoylation motif followed by a potential cleavage site predicted by the translated protein sequence (9), we speculated that AD7c-NTP could be secreted, and therefore, increased expression or accumulation of AD7c-NTP protein AD might be detected in cerebrospinal (CSF) and ventricular (VF) fluid. Initially, we performed Western blot analysis of postmortem VF from AD and aged control patients, and observed higher levels of both the ~41 kD AD7c-NTP protein and the ~18–21 kD AD7c-NTP related molecule in the AD samples. The studies were extended to measure AD7c-NTP content in VF from a large number of cases in which the postmortem diagnoses were certain. To do this, we employed our highly sensitive and reproducible AD7c-NTP ELSIA which detects only the 41 kD AD7c-NTP species (24, 25).

Using the AD7c-NTP ELSIA, the levels of AD7c-NTP protein measured in control VF samples were uniformly low, whereas in confirmed cases of AD, the VF levels of AD7c-NTP were more broadly distributed, and the mean values were significantly higher than control. Importantly, 84 percent of the AD cases had VF levels of AD7c-NTP that were higher than the maximum levels detected in the control group. These results are concordant with the findings in brain tissue homogenates analyzed using the same assay, and provide evidence that the AD7c-NTP protein may be secreted into CSF. Alternatively, the increased levels of AD7c-NTP protein in postmortem VF could reflect increased release following cell death. With either interpretation, the results suggest that the levels of AD7c-NTP protein present in CSF corresponds with the levels in brain tissue, and therefore could serve as an in vivo biomarker of AD-type neurodegeneration.

5.2. Elevated levels of AD7c-NTP in CSF from patients with early or moderately severe AD (9)

To determine the utility of such an assay for assessing patients with early or moderate degrees of cognitive impairment, we utilized the AD7c-NTP ELSIA to measure AD7c-NTP levels in clinical samples of CSF from patients in which detailed follow-up information had been systematically recorded. This study was possible only because CSF samples that had been prospectively banked by the Alzheimer’s Disease Research Center at the Massachusetts General Hospital were made available. Lumbar puncture CSF samples were obtained 5 to 10 years prior to the patient’s death, and in all cases, detailed clinical evaluation and postmortem follow-up results were available to confirm the diagnosis. In addition to the AD cases, AD7c-NTP levels were measured in CSF from patients with Parkinson’s disease (PD), multiple sclerosis (MS), normal aging, or non-relevant neurological diseases, e.g. chronic spondylosis. The value of this study was enhanced by the fact that all of subjects had been evaluated by detailed neurological, and in many instances, neuropsychiatric tests.

More than 300 CSF samples were analyzed under code using the AD7c-NTP ELSIA. The major findings were: 1) that the mean levels of AD7c-NTP in CSF were nearly three times higher in the AD than control group; and 2) the CSF AD7c-NTP levels were elevated above 2 ng/ml in 89 percent of the early AD cases versus 11% of the age-matched control cases. Blessed dementia scale (BDS) scores were used for correlation analysis of CSF AD7c-NTP levels with dementia. Significantly higher BDS scores were observed in the AD group, and a significant positive correlation was observed between AD7c-NTP levels and BDS scores, but not age, gender, or duration of disease. Stepwise multivariate regression confirmed the significant correlation between AD7c-NTP levels and cognitive impairment but not other variables. One of the most interesting results obtained from our work was that elevated levels of AD7c-NTP protein were detectable in CSF from patients in their early stages of AD (9).

Although the number of completely normal controls included in this study was limited due to the lack of detailed neurological and neuropsychiatric test results and follow-up clinical assessments of subjects in this category, the non-relevant neurological disease controls were also found to have low levels of AD7c-NTP protein in CSF. The patients with chronic, inactive multiple sclerosis (N=40), multi-infarct dementia (N=3), or Creutzfeld-Jakob disease (N=4) had mean CSF levels of AD7c-NTP protein that were similar to the normal control levels. In addition, the majority of patients with Parkinson’s disease (N=56), diffuse Lewy body disease (N=8), or Pick’s disease (N=7) had normal levels AD7c-NTP in lumbar CSF samples (9). Of interest is that several patients initially diagnosed with PD or DLBD and found to have elevated lumbar CSF...
levels of AD7c-NTP, were subsequently demonstrated to have cognitive impairment, and postmortem examinations revealed co-existing AD neurodegeneration. Independent studies utilizing an entirely different population base have confirmed these results (23, 24, 30). Therefore, elevated levels of AD7c-NTP in CSF appears to correlate with presence and severity of AD in the early and intermediate stages of disease. The value of the AD7c-NTP assay was further demonstrated by the results of another more recent independent study in which the high degrees of sensitivity and specificity of the AD7c-NTP ELSIA were confirmed, and the CSF levels of AD7c-NTP were found to correlate with both the severity of dementia and the phospho-tau concentrations in CSF from patients with AD (30).

As would be the case for all biomarkers of disease, it is important to recognize their potential limitations and pitfalls because such surrogate markers are diagnostic aids that should be used to guide further investigations needed to confirm or exclude a specific diagnosis. An important caveat regarding the use of AD7c-NTP as a biomarker for detecting AD is that conclusions drawn from the assay results should be based upon stably elevated levels of the protein in CSF or urine (see below). We have observed transiently elevated levels of AD7c-NTP protein in urine following acute injury due to cerebral infarction of a major neurosurgical procedure, but with subsequent analyses, the levels of AD7c-NTP were found to be similar to normal controls. In contrast, repeated assays of CSF from patients with bona fide AD revealed either stably elevated or progressive increases in the levels of AD7c-NTP protein. Therefore, a suspicion of AD should be based upon the detection of increased concentrations of AD7c-NTP in at least two separate samples of CSF or urine that preferably were obtained several months apart.

5.3. Elevated Levels of AD7c-NTP in Urine of Patients with Early AD

The size of the AD7c-NTP protein is sufficiently small to be excreted with urine. Using the ELSIA, significantly elevated levels of AD7c-NTP protein were detected in urine (24-hour collection with concentration) from patients with early or moderately severe AD compared with the levels measured in urine from normal aged controls (23). Like the CSF-based assay, the degree to which the AD7c-NTP ELSIA levels were elevated correlates with the severity of cognitive impairment. Moreover, the sensitivity and specificity of the urine-based ELSIA were found to be comparable to those of the CSF-based assay. These studies provided the first steps toward developing a completely non-invasive biomarker assay to aid in the detection of early AD.

5.4. Second generation novel assay to measure AD7c-NTP levels in CSF or urine

Although the AD7c-NTP ELSIA has the sensitivity and specificity required of a potential AD biomarker, the mechanics of the assay are somewhat labor-intensive and cumbersome due to the need for considerable quantities of a uniform batch of recombinant protein used to generate the standard curves and measure AD7c-NTP concentrations in biological fluids. Purification of intact recombinant AD7c-NTP protein is an arduous task due to spontaneous aggregation and formation of fibrillar precipitates. More recently, a highly simplified assay termed, “7C Gold”, was developed (22) to quantify the levels of AD7c-NTP protein in urine or CSF. The 7C Gold test (currently termed, “AD Alert”), is a gold particle monoclonal anti-AD7c-NTP based liquid phase immunoassay. Using the 7C Gold assay, the mean levels of AD7c-NTP protein were found to be three times higher in the AD than in aged control urine samples. Moreover, the 7C Gold assay has a sensitivity of 91% and specificity of 96.8% for detecting AD based upon analysis of 58 AD and 30 control samples (22). Reliable detection and quantification of AD7c-NTP protein in urine requires that the urine be sterile and free of particulate debris. In addition, the urine must be concentrated prior to analysis. The implications for a urine-based assay are that one could use it as a non-invasive screening test that should be repeated at later dates to confirm that the levels of AD7c-NTP protein in urine are chronically elevated. Positive results could be used to justify further detailed investigations, including analysis of CSF, brain imaging, and formal cognitive testing. The results could also be used to encourage participation in therapeutic clinical trials and monitor the effectiveness of novel regimens.

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7. REFERENCES

7. de la Monte, S. M., T. Luong, T. R. Neely, D. Robinson, and J. R. Wands: Mitochondrial DNA damage as a...
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