

Human Kallikrein 6 as a Biomarker of Alzheimer's Disease

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Background: Alzheimer's disease (AD) is a major cause of dementia in the elderly. It is generally difficult to diagnose accurately early AD. A few biomarkers, including τ protein and amyloid β -42, are now used as aids for diagnosis and monitoring of AD. Our aim was to examine the possible use of cerebrospinal fluid, blood and tissue, and human kallikrein 6 (hK6) concentration as a marker of AD.

Methods: We have used a highly sensitive and specific immunofluorometric procedure for measuring hK6. We measured hK6 in tissue extracts from AD brain or normal individuals, in cerebrospinal fluids of AD patients or normals and in whole blood of AD patients and normals and compared the findings. We have used ten pairs of AD/normal controls in all cases.

Results: We found that hK6 concentration in tissue extracts from AD brain were approximately twofold lower than extracts from normal controls. Further, we found that cerebrospinal fluid hK6 concentration is approximately a threefold increase, in comparison to cerebrospinal fluid controls ($p = 0.001$). We have also found that the whole blood hK6 concentration in AD patients is about ten times higher than hK6 concentration in normal controls ($p = 0.002$). We have immunohistochemically localized the expression of hK6 in epithelial cells of the chorioid plexus.

Conclusions: This is the first report describing significant elevations of cerebrospinal fluid and plasma and whole blood hK6 concentration in AD patients, in comparison to controls. These data suggest that hK6 may constitute a new biomarker for diagnosis and monitoring of AD. Copyright © 2001 The Canadian Society of Clinical Chemists

KEY WORDS: Alzheimer's disease; human kallikreins; brain; cerebrospinal fluids; diagnostic tests; human kallikrein 6.

Introduction

Alzheimer's disease (AD) is the major cause of dementia in the elderly. Although rare genetic forms of AD exist, most patients are classified as having sporadic AD, because no family history is usually identified. Pathologically, AD is character-

ized by neuronal and synaptic degeneration with an increased number of senile plaques and neurofibrillary tangles compared to nondemented individuals of comparable age (1–3).

The senile plaques, characteristic of AD, are composed of a central core of aggregated β -amyloid, a breakdown product of amyloid precursor protein (APP) (2). The neurofibrillary tangles are insoluble intracellular threadlike structures made up of a hyperphosphorylated form of a protein called τ , which is associated with microtubules (4).

Early and accurate diagnosis of AD is very important because early intervention may delay or arrest the reversible neuronal damage. Clinical diagnosis is not always accurate because the criteria are relatively subjective and the disease needs to be differentiated from other dementia illnesses (5–8). There is a need for developing biochemical diagnostic markers that could aid in the diagnosis of early AD and also for monitoring treatment.

Cerebrospinal fluid (CSF) is one of the preferred clinical samples for biomarker analysis in AD because it is in direct contact with the extracellular space of the brain. The discovery of serum or urine biomarkers for this disease is more preferable, due to the ease of collecting blood or urine, in comparison to CSF. Currently, two biochemical markers are used as aids for diagnosing AD, including CSF-total τ and CSF-amyloid β -42 ($A\beta$ -42) (9–25). The latter is a major component of the senile plaques, and the former is a component of intracellular neurofibrillary tangles. In AD, CSF-total τ is increased and CSF- $A\beta$ -42 is decreased. These two markers, either alone or in combination, are quite useful, but by no means 100% sensitive or specific for AD. Moreover, serum analysis of these biomarkers for diagnosis and monitoring is not particularly useful. More recently, another biomarker, neuronal thread protein and its derivatives, appears to be increased in CSF and urine of patients with AD, but its sensitiv-

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ity and specificity are not perfect either (26–29). For these reasons, there is a need for developing new biomarkers for serum and CSF analysis, for diagnosis and monitoring of AD.

Human kallikrein 6 (hK6, encoded by the KLK6 gene) is a new member of the kallikrein gene family, which is also known as zyme/protease M/neurosin (30–33). This is a secreted serine protease that has recently been found in many biologic fluids and tissues (34). Little *et al.* have reported that this protein may have amyloidogenic potential in the brain and may contribute to the pathogenesis of AD (30). Until recently, no methods were available for the quantitative measurement of hK6 in biologic fluids and tissues. We have developed a quantitative immunologic assay that is sensitive and specific enough to quantify hK6 in serum and CSF (34). We here examine the concentration of hK6 in brain tissue extracts obtained from AD brains or control subjects as well as the analysis of serum and CSF from AD patients or control subjects. We found significant differences in hK6 concentration in both the tissues and the two biologic fluids. We propose here that hK6 analysis in CSF and serum may aid in the diagnosis of AD.

Materials and methods

IMMUNOFLUOROMETRIC ASSAY FOR hK6

The details of this immunofluorometric assay have recently been described (34). The assay utilizes two hK6-specific polyclonal antibodies, one raised in mouse and the other raised in rabbit. This is a noncompetitive immunofluorometric procedure that incorporates the principles of time-resolved fluorometry for detection. The assay measures hK6 in the range of 0.5 to 200 $\mu\text{g/L}$ with precision of less than 10%. All tissue extracts, serum samples, and CSFs, were measured at various dilutions to bring the concentrations within the measuring range of the assay.

CLINICAL SAMPLES

For this preliminary investigation, we have used 10 whole blood samples obtained from patients with histologically confirmed (postmortem) AD, and 10 whole blood samples from normal individuals. Both series of samples were stored at -80°C until analysis. Because these whole blood samples were hemolyzed, we have centrifuged them; and the supernatants were diluted 10-fold in a 60 g/L bovine serum albumin solution to minimize the effect of hemolysis.

We have also used 10 cerebrospinal fluid samples from patients with confirmed AD and 10 cerebrospinal samples that were collected for investigation of infectious brain pathologies, but tested negative. The CSF samples were analyzed after 100-fold dilution in the diluent described above.

We have also used 10 brain tissue specimens obtained from the frontal cortex of patients with

confirmed AD and 10 frontal cortex brain specimens obtained from patients without AD. These tissues were pulverized on dry ice to fine powders and they were extracted for preparation of cytosolic extracts, essentially as described elsewhere (35). The tissue extracts were centrifuged and the supernatants used for hK6 analysis as well as measurement of total protein. These extracts were analyzed after 100-fold dilution in the diluent described above. The tissues, CSF, and blood, were obtained from the same ten individuals; their ages ranged from 61 to 91 with a median of 86 yr. There were two males and eight females in this series. The ages of the control subjects (for tissues) ranged from 44 to 81 with a median of 71 yr. There were five males and five females in this series.

The tissues and fluids from AD patients and tissues for the control subjects were obtained from the Institute for Brain Aging and Dementia Tissue Repository, University of California, Irvine, CA. These specimens were collected within 3.5 to 6.5 h postmortem and were immediately frozen for future use.

Results

Our results are summarized in Table 1. We found that hK6 content of frontal cortex tissue extracts from AD brains was reduced, in comparison to tissue extracts from control subjects. The average decrease was approximately twofold and the differences were of borderline statistical significance ($p = 0.05$ – 0.07 by *t*-test or Mann–Whitney test). We further observed a statistically highly significant increase of hK6 concentration in CSF of patients with AD, in comparison to the control subjects (about threefold; $p = 0.001$; Mann–Whitney test). Further, we observed a highly statistically significant increase of hK6 concentration in whole blood of AD patients, in comparison to control subjects (about 10-fold; $p = 0.002$; Mann–Whitney test).

Though the whole blood concentration of hK6 in all AD patients was less than 61 $\mu\text{g/L}$ and less than 9 $\mu\text{g/L}$ in all control subjects, one patient had a whole blood hK6 concentration of 539 $\mu\text{g/L}$. Because of the extremely high value of hK6 in this patient, we have fractionated this whole blood on a size-exclusion gel filtration column to establish the immunoreactive species and the molecular weight of the detected analyte. In Figure 1, it is shown that the whole blood of this patient contains a single immunoreactive species of an approximate molecular weight of 30 kDa, which is consistent with the molecular weight of free hK6. We have previously shown that the same molecular weight corresponds to the immunoreactive species of hK6 in serum, CSF, and seminal plasma and milk of lactating women (34).

Because hK6 concentration is quite high in CSF, we have attempted to immunohistochemically localize the source of hK6 in CSF. Various brain sections (formal infixed, paraffin-embedded tissue) were

TABLE 1
Comparison of hK6 Concentration Between Alzheimer's Disease and Control Subjects

	hK6 (ng/mg)				<i>p</i> (<i>t</i> -test)	<i>p</i> (Mann–Whitney)
	Range	Mean	SD	Median		
Tissue Extracts (n = 10)						
Alzheimer's disease	26–153	50	37	38		
Controls	4–261	100	74	94	0.07	0.05
Cerebrospinal Fluids (n = 10)						
		hK6, μg/L				
Alzheimer's disease	1110–3124	2121	762	1969		
Controls	141–1662	751	512	749	<0.001	0.001
Whole Blood (n = 10)						
		hK6, μg/L				
Alzheimer's disease	0–539	81	162	30		
Controls	0–9.1	3.6	3.0	3.0	ND ¹	0.002

ND: Not done; non-Gaussian distribution of data.

stained with a polyclonal anti-hK6 rabbit antibody and then detected by using classical immunohistochemical techniques. The highest concentration of hK6 was found in luminal cells lining the chorioid plexus (Figure 2). It is thus possible that the bulk of hK6 production occurs in this cell type.

Discussion

The KLK6 gene encodes for a serine protease (hK6) that is highly expressed in the central nervous system, as well as in many other organs (33). Recently, this secreted serine protease was identified in tissue extracts and various biologic fluids, including serum, nipple aspirate fluid, breast cyst fluid, seminal plasma, amniotic fluid, and breast cancer

cytosols (34). The biologic role of this serine protease in the central nervous system and other peripheral organs is currently unknown. It has previously been reported that hK6 has amyloidogenic potential and may contribute to the pathogenesis of AD (30). However, no data currently exist, comparing levels of hK6 in tissues or CSF between AD patients and normal controls. The availability of a highly sensitive method for measuring hK6 in biologic fluids enabled us to perform this study (34). Our data, summarized in Table 1, demonstrate that the concentration of hK6 in tissue extracts is reduced in AD patients, in comparison to control subjects. Additionally, there is dramatic increase of hK6 concentration in the CSF and blood of AD patients, in comparison to control subjects. We recognize that this is a relatively small study and that these results are preliminary. If confirmed with a larger group of patients, these data demonstrate that hK6 has potential as a new biomarker for diagnosis and monitoring of AD. Although we do not have experimental data, we speculate that the bulk of serum concentration of hK6 originates from diffusion of hK6 from the CSF. We further postulate that the increased concentration of hK6 in CSF of AD patients is responsible for the increased serum concentration of hK6 in these patients. In this small group of patients, we did not find a statistically significant correlation between CSF and whole blood hK6 concentrations, and one patient with AD had extremely high whole blood hK6 levels (Figure 1). It is thus possible that the diffusion of hK6 from CSF to the blood may not solely depend on the concentration gradient between these two fluids but also on other factors, including permeability of the blood–brain barrier. These suggestions need further investigation.

We do not know if the observed decrease of tissue hK6 content and the increase in CSF concentration of hK6 are interrelated and if the relative depletion of tissue hK6 is reflected by increases of hK6 in CSF. Further, we have immunochemically localized hK6

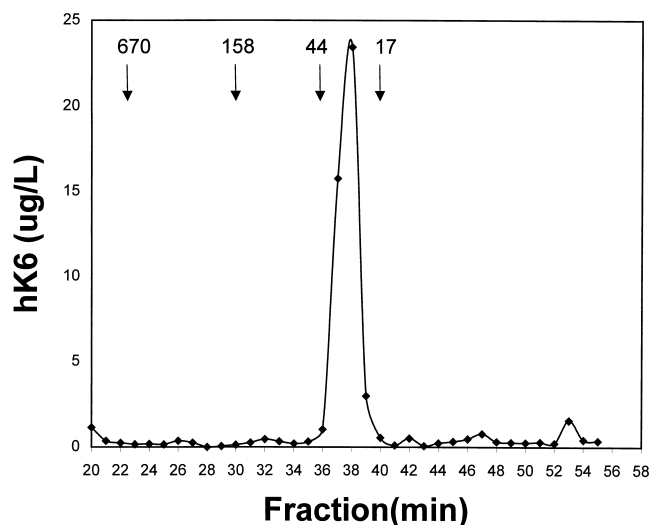


Figure 1 — High-performance liquid chromatography on a gel filtration column. Sample is whole blood of a patient with AD with extremely high blood hK6 concentration (539 μg/L). There is a single immunoreactive peak eluding at fraction 38 ± 1 , which corresponds to a molecular weight of ~30 kDa. These data support the view that hK6 in whole blood is present in its free, uncomplexed form.

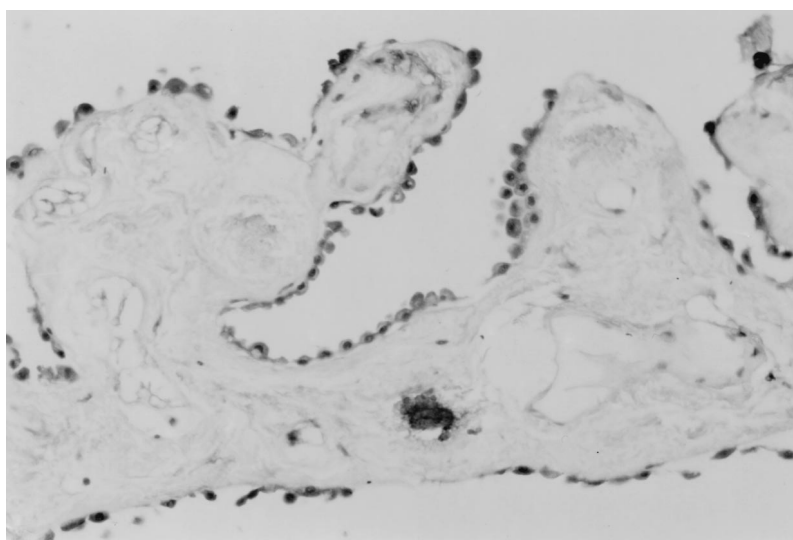


Figure 2 — Immunohistochemical localization of hK6 in paraffin-embedded formalin-fixed brain tissue. The figure reveals intense cytoplasmic positivity of luminal aspects of the lining cells of the choroid plexus (original magnification 400×).

production in the epithelial cells of the choroid plexus, but we do not as yet have functional hypotheses as to why hK6 is overproduced or is increasingly leaking into the CSF from adjacent diseased tissues.

We here provide the first indication that hK6 may be a new valuable biomarker for diagnosis and monitoring of AD. These data, if confirmed with a larger study, may have implications for the early diagnosis and monitoring of AD and may contribute to slowing the progression of this disease by allowing early diagnosis and administration of effective treatments.

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