

Decreased concentration of human kallikrein 6 in brain extracts of Alzheimer's disease patients

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Abstract

Background: The human kallikrein 6 gene (KLK6) encodes for a secreted serine protease, hK6, which is highly expressed in brain. Previous reports have associated hK6 with the pathogenesis of Alzheimer's disease. Our objective was to develop a highly sensitive immunoassay for hK6 and use it to examine the levels of hK6 in brain tissue extracts from Alzheimer's disease patients and control subjects.

Methods: We developed antibodies against hK6 and constructed a 'sandwich' type immunoassay. We then assessed levels of hK6 in brain extracts from normal individuals and patients with Alzheimer's disease.

Results: The hK6 assay was developed using a combination of two antibodies (a mouse monoclonal and a rabbit polyclonal). Purified recombinant hK6 was used as a calibrator. The detection limit of the assay was 0.05 $\mu\text{g/L}$. The intra and inter-assay coefficient of variation was less than 6.5%. We found no detectable cross-reactivity by the homologous proteins hK2, hK3, hK8, hK10, hK11, hK13 and hK14. The hK6 concentration in human brain tissue extracts from healthy ($n = 24$) and Alzheimer's patients ($n = 55$) were 10.1 ± 1.0 and 3.39 ± 0.26 $\mu\text{g/g}$ of total protein (mean \pm SE), respectively ($p < 0.001$). Similar differences were seen when the tissues were stratified by brain region (occipital, parietal, frontal and temporal cortex).

Conclusions: We conclude that the newly developed hK6 immunoassay is suitable for quantification of hK6 protein in biologic fluids and tissue extracts. The brain of Alzheimer's disease patients contains significantly less hK6 than the brain of nonaffected individuals. The possible connection of hK6 with the pathogenesis of Alzheimer's disease merits further investigation. © 2002 The Canadian Society of Clinical Chemists. All rights reserved.

Keywords: Human kallikreins; Serine proteases; Time-resolved fluorescence; Alzheimer's disease; Human kallikrein 6; Zyme

1. Introduction

Mammalian serine proteases are involved in many biologic functions including coagulation and fibrinolysis, digestion, activation or inactivation of hormones, receptors, cytokines, etc. The human kallikrein gene family consists of 15 serine protease genes (designated KLK1–KLK15) which share significant sequence homologies at the DNA and amino acid level (40–80%). Many of these genes are regulated by steroid hormones [1]. The human kallikrein 6 gene (KLK6), encodes for a secreted serine protease (hK6) [2].

hK6 has been identified by several laboratories and designated as zyme [2], protease M [3] or neurosin [4]. New nomenclature has now been adopted for human kallikrein genes [5]. The presence of aspartate in the binding pocket of hK6 predicts that this protein will produce trypsin-like cleavage. hK6 is synthesized as an inactive zymogen and is converted to an active enzyme by cleavage between Lys 21 and Leu 22 [4].

Messenger RNA encoding hK6 can be detected in some mammalian species but not in mice, rats or hamsters [2]. This gene is down regulated at metastatic breast cancer sites and is up-regulated in a subset of primary breast and ovarian tumors [3]. Serum hK6 concentration has been proposed as a biomarker for ovarian carcinoma [6]. hK6 is up-regulated in the breast carcinoma cell line BT474 by estrogens and

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progesterins, and to a lesser extent by androgens [7]. This gene is highly expressed in brain tissue, including cerebellum and spinal cord and also, in kidney, spleen, mammary tissue and salivary gland [7].

Brain serine proteases are implicated in synaptic plasticity, developmental processes, neurite outgrowth and in neurologic disorders including Alzheimer's disease (AD) [8,9]. These actions may be mediated by the proteolytic cleavage of zymogen precursors and propeptides, the activation of specific cell surface receptors and/or by the degradation of extracellular matrix proteins [10–12]. hK6 has been proposed to have amyloidogenic potential in the brain and may play a role in the development and progression of AD by cleaving *APP* (amyloid precursor protein), along the amyloidogenic pathway [2].

Four genes have been implicated to date in familial forms of AD. Three of them, when mutant, cause autosomal dominant forms of the disease (β *APP*, presenilin 1, and presenilin 2) and one in which a naturally occurring polymorphism (ApoE4) represents a major risk factor. ApoE is associated with late onset AD, while the 3 other genes are associated with highly penetrant early onset AD. Despite the genetic heterogeneity, all four genes have been shown to increase the production and/or deposition of amyloid beta peptide in brain, triggering AD-related neuronal degeneration [13]. The pathologic hallmark of AD is the deposition of amyloid as cerebrovascular, diffuse and neuritic plaques (within the brain extracellular space) and neurofibrillary tangles (within neurons). The regions that are most affected are the hippocampus and cerebral cortex. The pathogenesis of AD is thought to involve the dysregulated expression or abnormal processing of *APP* [14,15].

In recent years two cerebrospinal fluid (CSF) biochemical markers have emerged. Increased levels of CSF-tau and decreased CSF levels of $A\beta_{42}$, are good markers for AD [16]. Direct measurements of $A\beta$ isoforms in postmortem brain tissue of patients dying with presenilin1-linked familial form of AD, also show marked increases in the amount of $A\beta_{42}$ compared to control brain tissue and to brain tissue from subjects with sporadic AD [17]. In the recent Consensus Report of the Working Group on 'Molecular and Biochemical Markers of Alzheimer's Disease', it was recognized that although many molecular and biochemical markers for AD have been proposed, none has achieved universal acceptance or, for that matter, met the proposed criteria for an ideal biomarker [18].

Given the tremendous interest on proteases in the development of Alzheimer's disease [14] and the recent demonstration of highly expressed levels of hK6 in various parts of human brain [7], we set out to develop an immunoassay and to examine and compare the levels of this protease in brain tissue of Alzheimer's disease patients and nonaffected controls. We hypothesize that hK6 and other serine proteases of the kallikrein family may play a role in the development and progression of Alzheimer's disease.

2. Methods

2.1. Recombinant hK6 protein production and purification

Human 293 cells transfected with a plasmid containing the 1.4 kb hK6 cDNA were subjected to selection by growth in G418 (400 μ g/mL) for 3 weeks, after which time stable transformants were isolated. A positive clone that secreted hK6 protein in the culture medium was chosen. Purification of hK6 from the concentrated cell culture supernatants was achieved by reverse-phase HPLC using a linear gradient of 0.1% trifluoroacetic acid/acetonitrile. The general purification protocol has been described elsewhere [2,19,20].

2.2. Production and characterization of monoclonal antibodies

Female Balb/c mice were immunized with recombinant hK6 protein. Splenocytes were fused with murine myeloma cells using standard hybridoma technology. Briefly, 100 μ g (200 μ L) of hK6 were diluted 1:1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for subsequent injections, and given subcutaneously, every 3 weeks, for three times. Two weeks after the third injection, the mouse was injected IP with aqueous hK6 and three days later it was sacrificed and its spleen removed. To generate monoclonal anti-hK6 antibodies, the splenocytes were fused with the Sp2/0 myeloma cells (ATCC) using polyethylene glycol (PEG) 1500. The fusion cells were cultured in 96 well plates in DMEM (Dulbecco's modified Eagle Medium; Gibco BRL, Gaithersburg, MD) containing 20% fetal calf serum, 200 mM glutamine, 1% OPI (oxaloacetic acid, pyruvic acid, insulin), and 2% HAT (hypoxanthine, aminopterin, thymidine; Sigma Chemical Co., St. Louis, MO) for selection at 37°C, 5% CO₂ for 10 to 14 days. The supernatants were collected and screened for positive clones using the following immunoassay. Sheep antimouse IgG, Fc fragment –specific antibody (Jackson ImmunoResearch, West Grove, PA) was immobilized on 96-well white ELISA plates. Tissue culture supernatants diluted 10-fold in a general diluent (containing 60 g/L BSA, 50 mmol/L Tris, pH7.80, and 0.5 g/L sodium azide) were applied to the plates, incubated for 1 h and washed x 6. Biotinylated recombinant hK6 protein was then added (5–10 ng/well), incubated for 1 h and washed. Finally, alkaline phosphatase –conjugated streptavidin was added and the alkaline phosphatase activity was detected with time-resolved fluorescence, as described [21]. The positive clones were expanded sequentially in 24 well plates and 6 well plates in complete media (reducing the fetal calf serum to 15% and changing the HAT to HT). Supernatants were further tested by performing IgG isotyping and clones were subjected to limiting dilution. The clones were then expanded in flasks to generate large amounts of supernatants in serum free-media (CD-1 media; Gibco BRL) containing 200 mM glutamine.

2.3. Purification of monoclonal anti-hK6 antibodies

Tissue culture supernatants containing monoclonal antibodies were diluted twofold in 20 mM sodium phosphate buffer (pH 7.0) and injected into a HiTrap Protein G column (Amersham Pharmacia Biotech, Piscataway, NJ). After the column was washed with 20 mM sodium phosphate, the antibodies were eluted with 0.1 mol/L citric acid buffer (pH 2.7). The eluted antibody solutions were then neutralized to pH 7.6 and dialyzed overnight in 0.1 mol/L sodium hydrogen carbonate solution.

2.4. Polyclonal antibodies for hK6 protein

Polyclonal antibodies were raised against recombinant hK6. Rabbits were immunized with purified recombinant hK6 and boosted according to standard procedures [22]. Bleeds were screened using the following immunoassay: Goat antirabbit IgG (Jackson ImmunoResearch) was immobilized on 96-well white microtiter plates. The rabbit serum was then applied to the plates at different dilutions ranging from 1:500 to 1:100,000. Biotinylated recombinant hK6 was then added (5–10 ng/well). Finally, alkaline phosphatase-conjugated streptavidin was added, and the alkaline phosphatase activity was detected with time resolved fluorescence [21]. Rabbit antisera were used, as described below, without further purification.

2.5. Immunofluorometric assay for hK6

2.5.1. Standard assay procedure

The purified anti-hK6 monoclonal antibody (Code# E24) diluted in coating buffer (containing 50 mmol/L Tris pH 7.80) was dispensed into a 96-well white polystyrene microtiter plate [100 μ L/500 ng/well] and incubated overnight at room temperature. The plate was then washed 3 times with washing buffer (containing 9 g/L NaCl and 0.5 g/L Tween 20 in 10 mmol/L Tris buffer, pH 7.40). Twenty-five μ L of hK6 calibrators (recombinant hK6 in 60 g/L BSA) or samples were applied into each well along with 50 μ L of assay buffer. The assay buffer was a 50 mM Tris buffer, pH 7.80, containing per liter 60 g of BSA, 0.5 mol KCl, 0.5 g Tween 20, 10 g bovine immunoglobulins, 100 mL goat serum and 25 mL mouse serum. The plate was incubated for 2 h on an orbital shaker to allow the hK6 molecules to bind the plate. The plate was then washed 6 times. Subsequently, the plate was incubated for 1 h with 100 μ L per well of a rabbit anti-hK6 polyclonal antibody, diluted 10,000-fold in assay buffer. The plate was then washed 6 times with washing buffer. 100 μ L of alkaline phosphatase-conjugated goat antirabbit antibody (Jackson ImmunoResearch) diluted 2,000-fold in assay buffer was added to each well, incubated for 30 min and washed 6 times as described above. Finally, 100 μ L of 1 mM diflunisal phosphate (DFP) diluted in substrate buffer (0.1 M Tris, pH 9.1, 0.1 M NaCl and 1 mM MgCl₂) was added into each well and incubated for 10 min.

One hundred μ L of developing solution (1 M Tris base, 0.4 mol/L NaOH, 2 mM TbCl₃, and 3 mM EDTA) was pipetted into each well and mixed for 1 min. The fluorescence was measured with a time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada), as previously described [21]. The calibration and data reduction were performed automatically.

2.6. Assay characteristics

2.6.1. Determination of the detection limit

hK6 calibrators of 0, 0.1, 0.5, 2, 10, 50 μ g/L were prepared by diluting purified recombinant hK6 protein in 60 g/L of BSA. These standards were used to determine the detection limit of the assay, by running 10 replicates of each and defining the mean \pm standard deviation.

2.6.2. Linearity

To determine the linearity of the hK6 immunoassay, serum samples were serially diluted 2, 4 and eightfold in general diluent and their hK6 concentrations were measured with the assay.

2.6.3. Comparison study

We used this assay and another one, based on two polyclonal antibodies [19] to measure the hK6 concentration in human serum samples from 42 subjects.

2.6.4. Recovery, precision, cross-reactivity

The validity of the immunoassay for quantification of hK6 was examined by recovery, precision and cross-reactivity tests. Recovery tests were performed in serum samples spiked with purified recombinant hK6. The precision of the assay was investigated by using four serum samples. The hK6 concentration of each sample was measured 12 times (intra-assay) in one plate and for 12 subsequent days (inter-assay). The cross-reactivity was examined by using purified recombinant hK2, hK3, hK8, hK10, hK13 and hK14 (prepared in-house) as samples.

2.7. Clinical samples

We used 55 brain tissues from patients with confirmed Alzheimer's disease and 24 brain tissues from age-matched patients without AD. All brain tissues were obtained from The Institute for Brain Aging and Dementia Tissue Repository at the University of California, Irvine, CA. The serum samples were leftovers from routine biochemical testing. All tissues and sera were stored at -80°C until use. The brain regions represented in our tissue collections are described in Table 3.

2.8. Human tissue cytosolic extracts

Human brain tissue (0.2 g) was pulverized on dry ice to fine powders. One ml of extraction buffer (50 mM Tris, pH

8.0, 150 mM NaCl, 5 mM EDTA, 10 g/L of NP-40 surfactant, 1 mM PMSF, 1 g/L of aprotinin, 1 g/L of leupeptin) was added to the tissue powders and incubated on ice for 30 min with repeated shaking and vortexing every 10 min. Mixtures were then centrifuged at 14,000 g at 4°C for 30 min. The supernatants (cytosolic extracts) were then collected.

2.9. Total protein assay

The tissue extracts were used for hK6 analysis as well as for measurement of total protein by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) using BSA (bovine serum albumin) as a standard.

2.10. Statistical analysis

The statistical analysis was performed by SAS software (SAS Institute, Cary, NC). The distributions of the hK6 levels were compared between Alzheimer and control groups using the nonparametric Mann-Whitney and Kruskal Wallis Tests. Relationships between hK6 levels and age were assessed by Spearman correlation coefficient. For all analyses, a *p* value of < 0.05 was considered statistically significant.

3. Results

3.1. Assay optimization

We produced high titer rabbit polyclonal antibodies against human hK6 protein. We have also identified one mouse monoclonal antibody which was highly specific for hK6. Both the polyclonal rabbit antibody and the mouse monoclonal antibody revealed a single 30 kDa immunoreactive band with cerebrospinal fluid as sample on Western blots. Furthermore, both antibodies stained specifically immunohistochemical sections of paraffin embedded tissues, reported in detail elsewhere [23]. For development of the sandwich-type immunoassay for hK6, we chose to immobilize the monoclonal mouse antibody and use the rabbit polyclonal antibody for detection. We further used an alkaline phosphatase-labeled goat antirabbit secondary antibody and detected the signal with enzymatically amplified time-resolved fluorescence, as previously described [21]. All steps and amount of reagents added were optimized so that the background signal was minimal and specific signal was highest. Our final assay configuration is described under "Methods".

3.2. Calibration curve and detection limit

We used purified recombinant hK6 to prepare calibrators in a 60 g/L bovine serum albumin solution. A typical calibration curve for this assay is shown in Fig. 1. The assay has

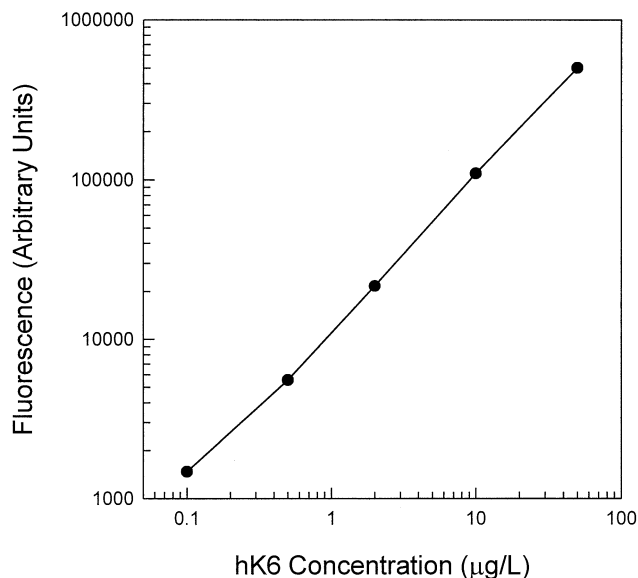


Fig. 1. Standard curve of the hK6 immunoassay. The background fluorescence (zero calibrator) has been subtracted from all other measurements.

a dynamic range from 0 to 50 µg/L. The detection limit, defined as the concentration of hK6 that can be distinguished from zero with 95% confidence, was 0.05 µg/L. This was calculated by adding fluorescence counts equal to 2 standard deviations of the zero standard (*n* = 12) to the fluorescence counts of the zero standard.

3.3. Precision, dilution linearity, recovery

We have tested the precision of this assay in both within-run and between-run assays (*n* = 12) using four different serum samples, with hK6 concentration between 1 and 6 µg/L. The coefficients of variation were < 6.5% in both cases. Furthermore, we analyzed three serum samples either undiluted, or diluted 2, 4 and eightfold. In all cases, we obtained linear dilution curves, with the measured values being within 10% of the expected concentrations from the

Table 1
Recovery of recombinant hK6 added in human serum samples

Serum Sample	hK6, µg/L			% Recovery
	Originally Present	Added	Recovered	
1	1.5	4.2	3.5	83
		8.3	7.2	86
2	2.5	4.2	3.2	76
		8.3	5.8	70
3	4	4.2	3.2	76
		8.3	6.7	81
4	2.3	4.2	3.5	83
		8.3	7.7	93
Control (BSA) ¹	0.0	4.2	4.1	97
		8.3	8.2	98

¹ Bovine serum albumin solution, 60 g/L.

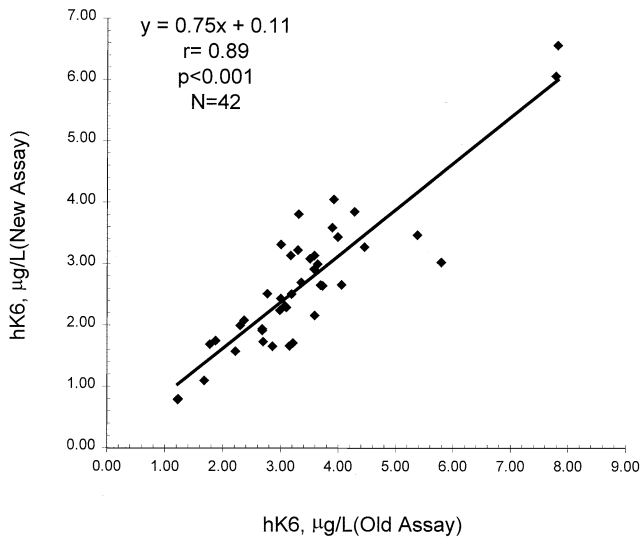


Fig. 2. Analysis of hK6 in 42 human serum samples with the old assay (x-axis) and the newly developed assay (y-axis). r = Pearson correlation coefficient. n = number of samples.

undiluted specimen. Recovery experiments in serum (Table 1) yielded recoveries ranging from 70 to 93%, with a mean of 81%.

3.4. Cross-reactivity

We tested the cross-reactivity of this assay against many homologous recombinant proteins prepared in-house, using a pichia pastoris expression system, as described elsewhere [20]. We found no detectable cross-reactivity with any of the available kallikreins, hK2, hK3 (PSA), hK8, hK10, hK11, hK13 and hK14, even when the cross-reactants were tested at concentrations as high as 1,000 $\mu\text{g/L}$.

3.5. Comparison with another method

The first method reported for hK6 analysis was based on a mouse polyclonal/rabbit polyclonal assay [19]. We have used the new assay and the previous assay to analyze 42 serum samples. The results (Fig. 2) show a good correlation between the two methods ($r = 0.89$).

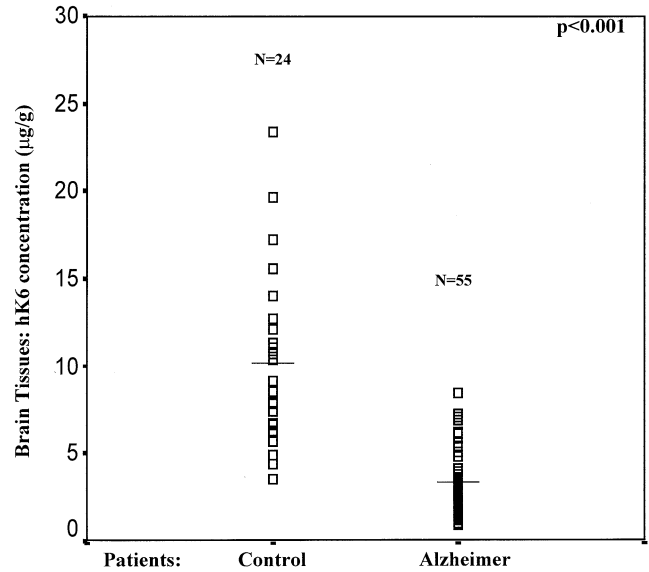


Fig. 3. hK6 content of Alzheimer’s disease and control extracts of brain tissues. The content is expressed as μg of hK6 per g of total protein. n = number of samples. $p < 0.001$ by the Mann-Whitney test. Horizontal lines indicate mean values.

3.6. Analysis of brain tissue extracts

By using the developed immunoassay, we analyzed brain tissue extracts from patients with Alzheimer’s disease or age-matched control subjects. These tissues were collected and stored from both groups in a similar manner, shortly after the patient’s death. We observed a highly significant reduction in hK6 content (approximately threefold) in brains from Alzheimer’s disease patients (Table 2 and Fig. 3). In Table 3 we report the data of hK6 content according to brain region. In control subjects, hK6 content does not differ significantly in various regions of the brain. The reduction in hK6 content in Alzheimer’s patients was seen in all regions, including occipital, parietal, frontal and temporal cortex.

4. Discussion

The human kallikrein 6 gene was cloned independently by three different groups of investigators from brain tissue

Table 2
hK6 content (in μg per g of total protein) of brain tissue extracts from Alzheimer’s disease and control subjects

Patient Group	Mean \pm SE ¹	Range	Percentiles					
			10	25	50	75	90	
Non-Alzheimer Brain Tissue								
hK6 ($\mu\text{g/g}$) (N=24)	10.1 \pm 1.0	3.5–23.4	4.6	6.4	8.9	12.5	18.5	
Alzheimer Brain Tissues								
hK6 ($\mu\text{g/g}$) (N=55)	3.4 \pm 0.3	0.9–8.4	1.4	1.9	2.8	5.0	6.9	

¹ SE, standard error.

Table 3
hK6 ($\mu\text{g/g}$) distribution in Alzheimer's disease patients and controls in different brain regions

Location	Number of samples	Mean \pm SE ^a	Median	Range	<i>p</i> value
Alzheimer Brain Extracts					
Occipital Cortex	5	3.9 \pm 0.80	3.50	2.30–7.00	0.016 ^b
Parietal Cortex	11	4.10 \pm 0.73	3.00	1.50–8.40	0.003 ^b
Frontal Cortex	21	4.13 \pm 0.69	3.70	0.90–15.5	0.002 ^b
Brain Stem	3	5.00 \pm 0.79	5.30	3.50–6.20	
Cerebellum	6	1.42 \pm 0.20	1.40	1.00–1.90	
Hippocampus	6	2.90 \pm 0.66	2.40	1.80–6.10	<0.001 ^c
Olfactory	3	1.73 \pm 0.26	1.70	1.30–2.20	
Meninges	1	2.50	—	—	
Choroid Plexus	1	2.10	—	—	
Temporal Cortex	1	2.70	—	—	
Non-Alzheimer Brain Extracts					
Occipital Cortex	5	10.04 \pm 1.70	10.11	4.87–14.04	
Parietal Cortex	6	11.77 \pm 1.66	11.23	6.27–17.23	0.401 ^d
Frontal Cortex	10	10.12 \pm 2.07	8.22	3.46–23.38	
Temporal Cortex	2	6.34 \pm 1.48	6.34	4.86–7.82	
Brain Stem	1	6.68	—	—	

^a Standard error

^b Between Alzheimer and Non-Alzheimer; Calculated by Mann-Whitney test

^c Cerebellum, Hippocampus and Olfactory versus Brain Stem, Occipital, Parietal and Frontal cortex; Calculated by Mann-Whitney test.

^d Between the non-Alzheimer groups; Calculated by Kruskal-Wallis Test

[2], breast tissue [3] or a colon carcinoma cell line [4]. The structure and genomic organization of this gene, as well as its tissue expression, are now well established [7]. The KLK6 gene encodes for a secreted serine protease which is highly homologous to other members of the kallikrein family, including prostate-specific antigen [1]. The precise biochemical function of this enzyme is not known but it has recently been shown that the protein is expressed at high levels by epithelial cells in many tissues [23]. hK6 is also found at high levels in cerebrospinal fluid and in human milk [19].

Until recently, no method was available for hK6 protein quantification. We have produced polyclonal antibodies against recombinant hK6 protein and used them to develop a first generation immunologic assay for hK6 [19]. By using this assay, we confirmed that hK6 is a secreted protein, found in various biologic fluids [19]. We have also provided preliminary evidence that hK6 concentration is lower in brain of Alzheimer's disease patients, in comparison to normal controls [24] and that the serum concentration of this protein is elevated in patients with ovarian carcinoma [6]. We have thus proposed that hK6 is a new biomarker for diagnosis and monitoring of ovarian cancer [6].

In this study, we describe the generation of monoclonal antibodies against hK6 and their incorporation into a monoclonal/polyclonal sandwich immunoassay for measuring hK6. This improved assay is ten times more sensitive than the previously described method and has the advantage of simplicity, availability of more consistent immunologic reagents and elimination of a secondary coating antibody which may facilitate nonspecific interactions with serum or tissue extract components. Validation of this assay indicates

that it is highly sensitive (detection limit of 0.05 $\mu\text{g/L}$), specific and precise. The lower recovery (81%) suggests that the added hK6 could interact with proteinase inhibitors, as is the case with PSA and hK2 [25]. However, we did not examine this further. This assay should be instrumental in further examining the biologic function and utilization of this molecule in diagnostics.

In accordance with our own preliminary findings [24], Ogawa *et al.* also reported reduction of hK6 transcripts and protein in two brains from Alzheimer's disease patients, in comparison to normal controls [9]. In this study, we report analysis of hK6 in brain extracts from 55 patients with Alzheimer's disease and 24 matched controls. With this significantly larger series of patients, we further confirm that hK6 levels in brain of Alzheimer's disease patients are reduced by approximately threefold, in comparison to controls. This decrease was seen in various brain regions, including the occipital, parietal, frontal and temporal cortex. We do not know if this reduction is related to the pathogenesis or is a consequence of Alzheimer's disease.

The role of many proteases in the pathogenesis and progression of Alzheimer's disease, including the recently discovered aspartyl proteinases, is well accepted [14]. Furthermore, the role of serine and other proteases in neural plasticity has also been reviewed [8]. Little *et al.* provided the first evidence that hK6 (also known as zyme) may be associated with Alzheimer's disease. We have previously examined the expression of all kallikreins in brain tissue and found that at least eleven of them are expressed in the brain [1]. It is very likely that hK6 and other kallikreins have a biologic function in this tissue. To this end, we speculate that the global reduction of the concentration of this enzyme

in brain tissue of Alzheimer's disease patients may be associated with the pathogenesis and progression of this disease. Particularly, it is interesting that in Alzheimer's disease, the concentration of this enzyme is decreased rather than increased, prompting us to speculate that it may have anti-amyloidogenic potential. It will be worthwhile to further examine the biologic function of this enzyme in brain and understand why in Alzheimer's disease, its concentration is significantly reduced. Recently, other groups reported that the amyloid deposition of Alzheimer's disease may be due to deficiency of proteolytic enzymes which are capable of digesting and clearing such deposits from brain. Two such enzymes are neprilysin [26,27] and insulin-degrading enzyme [28].

In conclusion, we report the development of an improved, highly sensitive immunoassay for human kallikrein 6 and demonstrate significant reductions in the concentration of this enzyme in brain tissue from Alzheimer's disease patients. The role of hK6 in the pathogenesis and progression of Alzheimer's disease merits further investigation.

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