Expression of the kallikrein gene family in normal and Alzheimer's disease brain

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The human kallikrein gene family consists of 15 serine proteases. We examined the expression of the kallikrein genes in human cerebral cortex and hippocampus by RT-PCR and compared their expression between Alzheimer's disease (AD) and control tissue. KLK1, 4, 5, 6, 7, 8, 10, 11, 13 and 14 are expressed in both cerebral cortex and hippocampus. KLK9 is expressed in cortex but not hippocampus, whereas KLK2, 3, 12 and 15 are not expressed in either tissue. We demonstrate an 11.5-fold increase in KLK8 mRNA levels in AD hippocampus compared to controls. The KLK8 gene product, neuropsin, processes extracellular matrix and is important for neuronal plasticity. Therefore, the increase in KLK8 could have detrimental effects on hippocampal function in AD. *NeuroReport* 12:2747–2751 © 2001 Lippincott Williams & Wilkins.

Key words: Cortex; Hippocampus; Human; mRNA; RT-PCR; Serine protease

INTRODUCTION

Proteases have been shown to play essential roles in the nervous system, including neurite outgrowth, synaptic plasticity, and neuronal degeneration. Proteases are also implicated in the etiology of Alzheimer's disease (AD), which is characterized by neuronal damage and loss of function. Serine proteases have been shown to participate in the processing of physiologically active macromolecules, digestion of extracellular matrix proteins, and cleavage of synapse-related proteins [1]. The kallikrein gene family is a subfamily of serine proteases. In humans, this gene family was, until recently, known to include only three members, KLK1 (coding for hK1, tissue kallikrein EC 3.4.21.35), 2 and 3 (coding for prostate-specific antigen, or PSA). Recent studies have identified up to twelve new members of the KLK family on chromosome 19q13.3-q13.4 [2,3].

Some of the new members of the kallikrein gene family reportedly perform important functions in nervous system injury and disease. KLK6 is up-regulated in the spinal cord after kainate injection [4], and in cerebrospinal fluid and serum in AD patients, in comparison to controls [5]. Little *et al.* [6] have provided evidence that the KLK6 gene product has amyloidogenic potential and might play a role in amyloid precursor processing in AD. One of the possible functions of the KLK7 gene product is to activate inter-

Table 1. Hippocampus samples used for RT-PCR.

| Sample no.	Age (years)	Gender	Postmortem delay (h)
Controls			
1	70	M	N.D.
2	75	M	2.5
3	75	M	3.5
4	77	M	6.5
5	74	F	7.75
6	66	M	8.0
7	79	M	N.D.
8	83	M	6.0
Means ± s.e.m.	74.88 ± 1.85		5.71 ± 0.92
AD			
9	79	F	7.5
10	56	F	8.0
11	91	F	5.4
12	84	M	3.4
13	77	M	2.25
14	75	M	3.0
15	66	M	3.0
16	77	F	2.25
17	73	M	4.0
18	72	M	2.25
Means ± s.e.m.	75.00 ± 3.01		4.11 ± 0.68

N.D. = not determined.

leukin-1β (IL-1β), which is over-expressed in AD and is involved in the inflammatory response to damage [7]. KLK8 mRNA is localized at high concentration in the neurons of hippocampus and amygdala and exhibits an activity-dependent regulation during epileptogenesis and long-term potentiation (LTP) [8–11]. The KLK8 gene product effectively cleaves fibronectin, a major extracellular matrix protein [12], which could account for its dramatic effects on plasticity [13]. In this study, we examined the expression of all 15 known members of the kallikrein gene family in the human cortex and hippocampus by RT-PCR and compared the expression of several of these genes between AD and control tissue.

MATERIALS AND METHODS

Human postmortem brain tissue: Hippocampal and parietal cortex tissue samples from normal, neurologically

unimpaired subjects ($n = 8$; one female, seven males) and samples from subjects with AD ($n = 10$; four females, six males) were provided by the Institute for Brain Aging and Dementia Tissue Repository at the University of California, Irvine. A diagnosis of AD was confirmed by pathological and clinical criteria [14]. Control and AD samples were matched for age (Table 1). Tissue was frozen at autopsy and stored at −80°C until use. For determination of differences in KLK8 mRNA levels between controls and AD samples, all hippocampal samples were used except for number 17. This sample had a very high KLK8 intensity that was > 2 s.d. above the mean, although all other measurements from this sample were within the expected range.

RNA isolation: Total cellular RNA was purified from hippocampal samples using the TRIzol™ Reagent (Gibco/
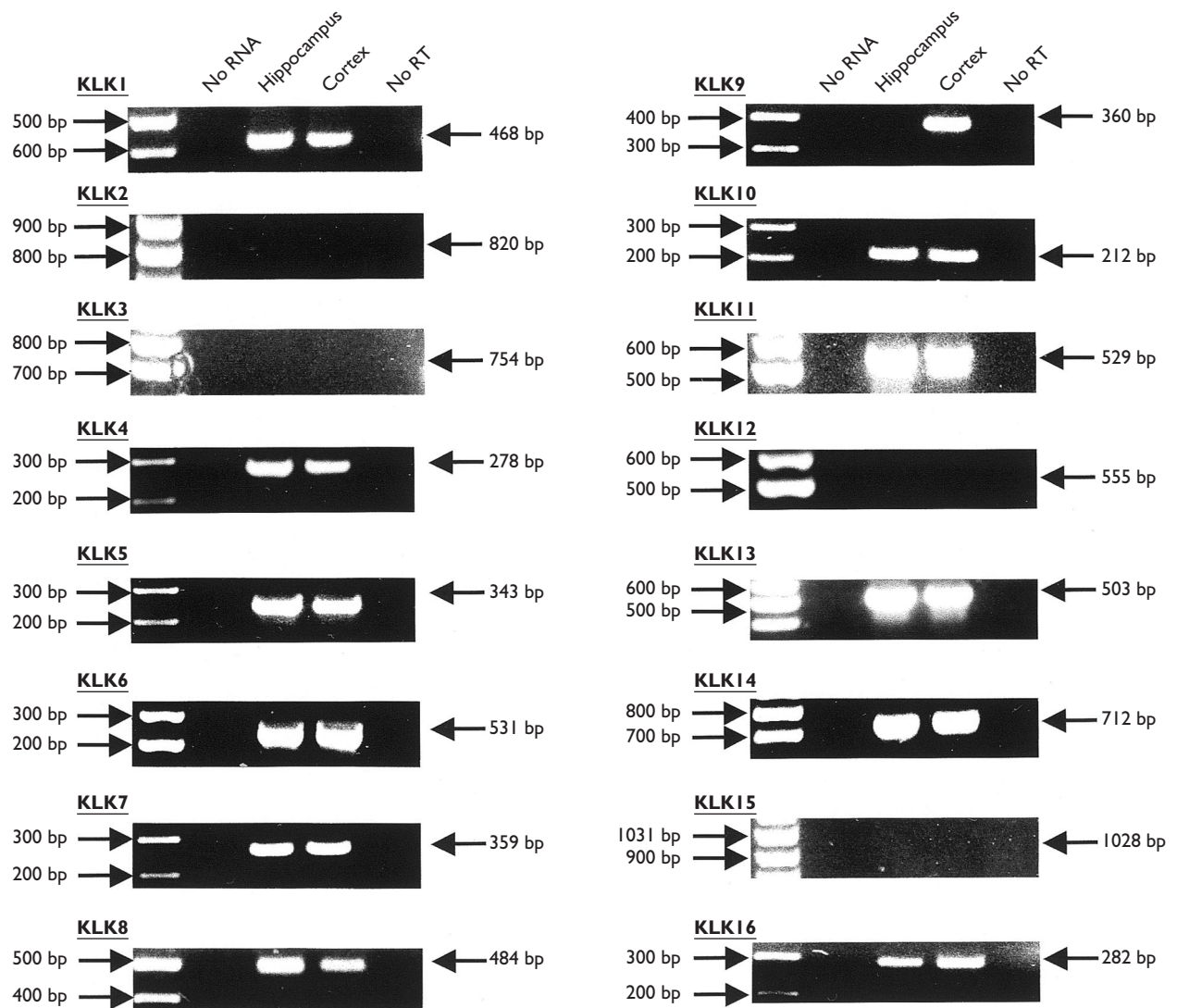
Fig. 1. Expression of the human kallikrein gene family in human cortex and hippocampus. All 15 kallikrein genes were analyzed by RT-PCR of RNA from control human cortex and hippocampus. Conditions were as described in Table 2. Negative controls were reactions containing no RNA or no reverse transcriptase (no RT). RT-PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

BRL, Burlington, ON, Canada) following the manufacturer's protocol. Briefly, 100 mg frozen tissue was homogenized in 1 ml TRIzol, and the homogenate was subjected to chloroform extraction, isopropanol precipitation, ethanol washes, and resuspension in 20 μl autoclaved, double-distilled water. Total RNA concentration and RNA purity were determined by absorbance at 260 and 280 nm using a Beckman DU-64 spectrophotometer. Samples exhibiting an absorbance ratio (260/280) ⩾ 1.65 and exhibiting strong 28S and 18S ribosomal RNA bands on 1% (wt/vol) agarose gels were used for further analysis. RNA samples were stored at −20°C until use.

*cDNA synthesis:* Human hippocampal total RNA (4 μg) was reverse transcribed into cDNA in 40 μl reaction mixtures containing 5 mM MgCl$_2$, 1× PCR buffer II (50 mM KCl, 10 mM Tris–HCl pH 8.3), 1 mM deoxynucleotide triphosphates, 1 U/μl RNase inhibitor, 2.5 μM oligo d(T)^{16} and 2.5 U/μl MuLV reverse transcriptase using the GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT) and the GeneAmp PCR system 400 thermal cycler (Perkin Elmer) at 37°C for 1 h followed by incubation at 99°C for 5 min to terminate the reaction and cooling at 4°C for 5 min.

PCR amplification: AmpliTaq Gold^{TM} DNA polymerase (Perkin Elmer) or HotStar^{TM} Taq polymerase (Qiagen, Mississauga ON, Canada) and [α^{32}P]dCTP (3000 Ci/mmol, Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) were used for amplification. The amplification involved a hot start at 95°C for 12–15 min, denaturation at 94°C for 30 s, annealing for 30 s, extension for 1 min at 72°C, and a final extension at 72°C for 7–10 min. For Fig. 1 we used 45 cycles. For Fig. 2, the optimal cycle number was determined for KLK4-KLK8, KLK10, KLK14 and β-actin to ensure that relative quantification was performed in the linear phase of amplification. Gene-specific primers, annealing temperatures and optimal cycle numbers where applicable for all of the kallikrein family genes are shown in Table 2. The primers were synthesized by the Central Facility of the Institute for Molecular Biology and Biotechnology (MOBIX) at McMaster University, Hamilton, Ontario, and were designed to cross intron–exon boundaries.

Analysis: Aliquots of 10 or 15 μl of each RT-PCR reaction mixture were analyzed by 1–2% (w/v) agarose gel electrophoresis and either ethidium bromide staining or phosphorimage analysis as noted in the figure legends. A 100 bp ladder (MBI Fermentas, Flamborough, ON, Canada) was used as a mol. wt marker. The amount of incorporated ^{32}P was determined using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Statistical comparisons between control and AD subjects were based on two-tailed Student's *t*-test calculated using Microsoft Excel 2000. Data are shown as mean ± s.e.m.

RESULTS

Kallikrein gene family mRNA expression in human brain tissue: We examined the expression of the kallikrein family in human cortex and hippocampus from control patients by RT-PCR. As shown in Fig. 1, KLK1, 4, 5, 6, 7, 8, 10, 11, 13 and 14 are expressed in both cortex and hippocampus. KLK9 is expressed in cortex but not hippo-
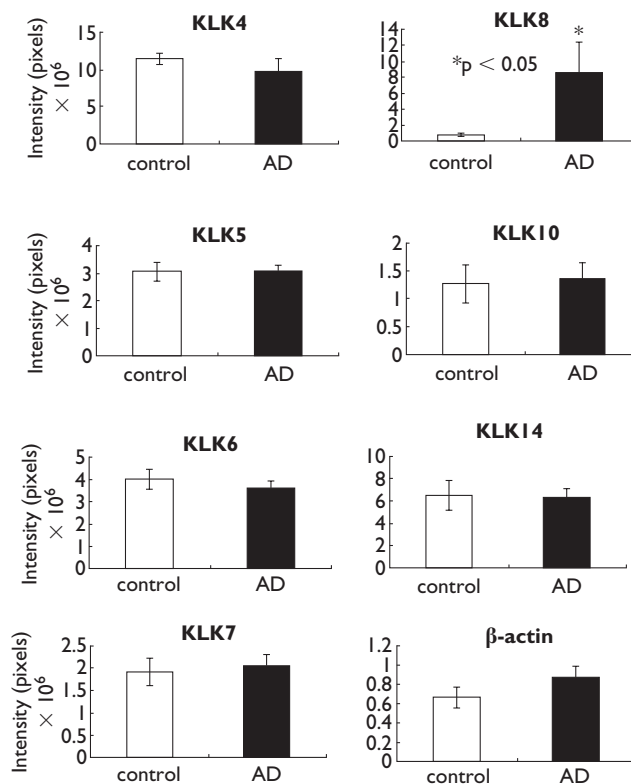
Fig. 2. Comparative RT-PCR of the kallikrein gene family in human control and AD hippocampus. RT-PCR products from 8 control and 10 AD samples were analyzed by RT-PCR, agarose gel electrophoresis and phosphorimage analysis. Bars represent means for each group. Error bars represent s.e.m.

campus, whereas KLK2, 3, 12 and 15 are not expressed in either tissue. Total RNA from human prostate was used as a positive control for RT-PCR of KLK2, 3 and 15. Also, total RNA from uterus was used as a positive control for RT-PCR of KLK12 (data not shown). Negative controls were reactions containing no RNA or no reverse transcriptase (no RT).

Samples: Eight control and 10 AD postmortem hippocampus samples (Table 1) were analyzed for relative levels of kallikrein gene family mRNA. The average age of the subjects in the control group was 74.88 ± 1.85 years and that of the AD group was 75.00 ± 3.01 years. The average postmortem delay was 5.71 ± 0.92 h for the control group and 4.11 ± 0.68 h in the AD group (*p* = 0.089). No significant differences were observed in the yield of total RNA extracted from either group (355.6 ± 29.2 μg/g tissue for control and 360.1 ± 36.4 μg/g tissue for AD samples; *p* = 0.464), or in the integrity of the purified RNA. Regression analysis revealed no significant correlation between postmortem delay and yield of total RNA (r = 0.211 for control and r = 0.219 for AD).

Comparison of kallikrein gene family mRNA levels between AD and controls: Of the 15 known members of the kallikrein gene family, 10 were expressed in cortex and hippocampus, regions of the brain that are severely

Table 2. Primers used for RT-PCR.

KLK	Aliases		Primer sequence[1]	Annealing temperature (°C)	Cycle number
1	Pancreatic/renal kallikrein	forward	TGGAGAACCACACCCGCCAAG	64	45
		reverse	ACGGCGACAGAAGGCTTATTG		
2	hGK2	forward	GGTGGCTGTGTACAGTCATGGAT	56	45
		reverse	CAGAAAGCACAGGTCAGTAGGAC		
3	PSA	forward	TGCGCAAGTTCACCCTCA	58	45
		reverse	CCCTCTCCTTACTTCATCC		
4	KLK-L1	forward	TGACCCGCTGTACCACCCCA	63	42
		reverse	GAATTCCTTCCGCAGGATGT		
5	KLK-L2	forward	GGATGCTTACCCGAGACAGA	58	37
		reverse	GCTGGAGAGATGAACATTCT		
6	Zyme	forward	TACCAAGCTGCCCTCTACAC	68	33
		reverse	ACAAGGCCTCGGAGGTGGTC		
7	SCCE	forward	GAATGAGTACACCGTGCACC	65	45
		reverse	TGCCAGCGCACAGCATGGAA		
8	neuropsin	forward	ACAGACCATGCCATCTGTGA	60	40
		reverse	ATGAGTGCCAACCCCATTC		
9	KLK-L3	forward	CATGCAGTGTCTCATCTCAG	63	45
		reverse	CTTCGGCCTCTCTTGGTCTT		
10	NES-1	forward	GCTTCCCTACCGCTGTGCT	62	42
		reverse	CACTCTGGCAAGGGTCCTG		
11	TLSP	forward	GGGAGAGACCAGGATCATCA	65	45
		reverse	TTCCTGCACGCTGGCACACA		
12	KLK-L5	forward	GGCTTCTCTGTGACCCATCC	61	45
		reverse	GGGGAAGTGATGGAGGAGAT		
13	KLK-L4	forward	CCAACATCCAACTTCGCTCA	58	45
		reverse	TTCAGGGATGCAACATCTGG		
14	KLK-L6	forward	CTGGCTATAGCCATGACACA	60	43
		reverse	TCGTTTCCTCAATCCAGCTT		
15		forward	CAAGTGGCTCTCTACGAGCG	64	45
		reverse	ATCACACGGGTGGTCATGTG		
β-actin		forward	TCATGAAGTGTGACGTTGACATC	64	18
		reverse	AGAAGCATTTGCGGTGGACGATG		

[1] All nucleotide sequences are given in 5'-3' orientation

affected in AD. We compared the expression of several of these genes between AD and control hippocampus. To control for variations in mRNA levels between samples, β-actin gene expression was used as an internal standard for each sample. As shown in Fig. 2, no statistically significant differences in levels of β-actin mRNA were observed between AD and control samples ($p = 0.11$). Furthermore, no statistically significant differences in levels of KLK4, 5, 6, 7, 10, or 14 mRNA were observed between AD and control samples (Fig. 2). However, an 11.5-fold increase in KLK8 mRNA was seen in the hippocampus of patients with AD compared to controls ($p = 0.038$, Fig. 2).

DISCUSSION

We examined the expression of the kallikrein gene family in the human cerebral cortex and hippocampus by RT-PCR and compared the expression of several of these genes between AD and control tissue.

We demonstrate here that KLK1, 4, 5, 6, 7, 8, 10, 11, 13 and 14 are expressed in both the cortex and hippocampus. KLK9 is expressed in the cortex but not the hippocampus, whereas KLK2, 3, 12 and 15 are not expressed in either tissue. KLK9 may exhibit a different and possibly more limited distribution pattern than other KLK gene family members in the brain. KLK 1, 4, 5, 6, 7, 9, 11, 12, 13 and 14 were previously reported to be expressed in the human brain [2,3,15], but only KLK8 and KLK11 mRNA were

known to be expressed in the hippocampus [3,8,16]. Our results increase the number of human kallikreins known to be expressed in cortex and hippocampus. Previous reports [3,17] indicated that KLK10 is not expressed in the brain. However, by using RT-PCR instead of northern blot analysis, we have demonstrated that KLK10 is expressed in both the cortex and hippocampus.

In this RT-PCR study, we used eight control and 10 AD age-matched hippocampal samples. We demonstrate an 11.5-fold increase in KLK8 mRNA levels in the hippocampus of patients with AD compared to controls. Although little is known about the function of the KLK8 gene product in the human brain, the critical role of murine KLK8 in normal hippocampal function has been demonstrated by examining deficits in dendritic morphology, electrophysiology and learning behavior of transgenic mice deficient in the KLK8 gene product, neuropsin [18]. Neuropsin, which is localized predominantly in the hippocampal CA1-CA3 subfields of mice, may be important in synaptogenesis and neural plasticity through proteolytic modification of pre- and post-synaptic structures. Neuropsin influences synaptogenesis in neural development [19], regulation in LTP [11], and seizure in kindled brain [9,20]. Since neuropsin shows activity-dependent changes in mRNA expression [8–12,20], it may be involved in the structural changes occurring during synaptic plasticity.

Murine neuropsin has been characterized as a secretory

protease that cleaves extracellular matrix proteins [12]. Recombinant mouse KLK8 protein effectively cleaves fibronectin [12], a major extracellular matrix protein necessary for neurite attachment and plasticity; the integrin-fibronectin interaction is important for learning and memory and for the induction of LTP [21,22]. High expression levels of KLK8 gene product affect hippocampal function by encouraging extracellular matrix degradation [11,12]. This may result in excessive retraction of neurites at synapses and inhibition of their rearrangement, leading to disruption of learning and memory in AD. Recent studies have provided evidence that α2-macroglobulin (α2M) is genetically associated with the pathogenesis of AD [23]. Interestingly, murinoglobulin I, which belongs to the α2M family, is a specific inhibitor of the mouse KLK8 gene product, neuropsin, in the brain [24]. These data strongly suggest that changes in KLK8 gene expression in the brain are pathologically associated with AD.

Our data fail to support a difference in KLK6 mRNA levels in AD hippocampus compared with control brains. The protein product of KLK6 is reportedly involved in processing of APP [6] and Aβ degradation [25]. KLK6 mRNA levels are decreased in AD brain in one report [25], but increased in AD brain in another [6]. hK6 protein is decreased in AD brain tissue but increased in cerebrospinal fluid, plasma and whole blood of AD patients compared to controls [5]. The widely differing results obtained by ours and the other groups may be derived from differences in tissue sampling, sample numbers or methods of analysis.

CONCLUSION

We have demonstrated the expression of 10 of the 15 human kallikrein genes in both cortical and hippocampal brain tissue. Of these 10 genes, we find one that exhibits altered expression in AD hippocampus, increased levels of KLK8 mRNA. The dramatic increase in KLK8 expression in AD could compromise hippocampal plasticity by promoting excessive modification of the extracellular matrix.

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