Blood-Based Biomarkers

A novel detection method of cleaved plasma high-molecular-weight kininogen reveals its correlation with Alzheimer's pathology and cognitive impairment

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Abstract

Introduction: Accumulation of β-amyloid is a pathological hallmark of Alzheimer's disease (AD). β-Amyloid activates the plasma contact system leading to kallikrein-mediated cleavage of intact high-molecular-weight kininogen (HKi) to cleaved high-molecular-weight kininogen (HKc). Increased HKi cleavage is observed in plasma of AD patients and mouse models by Western blot. For potential diagnostic purposes, a more quantitative method that can measure HKc levels in plasma with high sensitivity and specificity is needed.

Methods: HKi/c, HKi, and HKc monoclonal antibodies were screened from hybridomas using direct ELISA with a fluorescent substrate.

Results: We generated monoclonal antibodies recognizing HKi or HKc specifically and developed sandwich ELISAs that can quantitatively detect HKi and HKc levels in human. These new assays show that decreased HKi and increased HKc levels in AD plasma correlate with dementia and neuritic plaque scores.

Discussion: High levels of plasma HKc could be used as an innovative biomarker for AD.

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Keywords: Alzheimer's disease; Contact system activation; HMW kininogen; Cleaved HMW kininogen; Plasma biomarker; Sandwich ELISA; Diagnosis; Dementia

1. Introduction

High-molecular-weight kininogen (HKi) is a key constituent of the plasma contact-kinin system [1]. Activation of factor XII (FXII) triggers HKi cleavage by plasma kallikrein, resulting in the generation of cleaved high-molecular-weight kininogen (HKc) and the release of the proinflammatory peptide bradykinin. Increased activation of the contact system has been reported in thromboinflammatory diseases such as ischemic stroke [2] and myocardial infarction [3], as well as in autoimmune diseases [4] and hyperlipidemia [5]. More dramatic HKi cleavage is observed in hereditary angioedema [6], systemic lupus erythematosus [7], cancers [8], and sepsis [9].

The Alzheimer's disease (AD)-associated peptide Aβ42 can activate the contact system *in vitro*, leading to FXII-dependent cleavage of HKi [10–13]. *In vivo*, wild-type mice intravenously injected with Aβ42 have increased plasma HKi cleavage, which is not observed in FXII-knockout mice [14]. Furthermore, increased plasma HKi cleavage is found in two different AD mouse models driven by overexpression of human β-amyloid (Aβ) [14,15], suggesting that elevated Aβ expression can lead to increased contact system activation. The idea that plasma FXII activation and HKi cleavage contribute to AD

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pathogenesis is supported by studies showing that depletion of plasma FXII using an antisense oligonucleotide reduces plasma HKi cleavage, lowers brain inflammation, and improves cognitive function in an AD mouse model [15]. Importantly, increased HKi cleavage and elevated plasma kallikrein activity are also found in the plasma of AD patients, with the amount of HKi cleavage correlating with an established marker of AD progression [14]. Because some AD patients show HKi cleavage without detectable FXII activation [14], changes in HKi and HKc levels might be a more sensitive indicator of possible AD status.

Cleavage of HKi to HKc can be detected by Western blot, but this technique lacks the sensitivity needed for a diagnostic tool and is not amenable to a clinical setting. A more quantitative method that can measure HKi and HKc levels in plasma with high sensitivity and specificity would therefore be of interest as a possible tool to aid in the diagnosis of AD.

Here, we developed three classes of monoclonal antibodies: (1) one detecting only HKi; (2) one detecting only HKc, and (3) one detecting both HKi and HKc (HKi/c). We then established two sandwich ELISAs capable of discriminating between HKi and HKc in human plasma. Finally, we demonstrated the ELISAs' ability to show differences in HKi and HKc levels between Aβ-activated plasma versus vehicle-treated plasma and in AD patient plasma versus nondemented (ND) control plasma. These tools may help in the diagnosis of AD and other diseases characterized by contact system activation.

2. Methods

2.1. Production of monoclonal antibodies

For immunization, 50 μg of keyhole limpet hemocyanin–conjugated peptide (IQSDDDWIPDIQIDPNGLSC), which maps to the unique light chain of HK but not low-molecular-weight kininogen or HKi- and/or HKc-purified protein from human plasma (molecular innovations), was injected into Armenian hamsters and mice. See Supplementary Methods for further experimental details.

2.2. Antibody screening

Supernatants from hybridomas were screened at The Rockefeller High Throughput Screening Resource Center by direct ELISA with or without a fluorescent substrate using purified human HKi and HKc. See Supplementary Methods for further details.

2.3. Cloning of hybridoma cells and purification of monoclonal antibody

Cloning of hybridoma cells and purification of monoclonal antibody were performed at the Antibody and Bioresource Core Facility at Memorial Sloan Kettering Cancer Center. Details are in Supplementary Methods.

2.4. Sandwich ELISAs for measuring HKi and HKc levels in human plasma

After the purification step, the HKi (2B7) and HKc (4B12) antibodies were biotinylated using EZ-Link® Sulfo-NHS-LC-Biotin (Thermo Scientific) according to the manufacturer's instructions. After coating Fisherbrand™ 96-well plates with HKi/c antibody (3E8) at 100 ng/well in 0.1 M sodium bicarbonate buffer pH 9.6 (binding buffer) overnight at 4°C, the plates were washed with 0.1% Tween-20/PBS and incubated in blocking buffer (1% BSA in 0.1% Tween-20/PBS). Plates were washed three times between each step thereafter. Then, HKi/c protein or human plasma diluted in blocking buffer was incubated for 60 minutes at room temperature. After incubation with biotinylated 2B7 or 4B12 for 60 minutes at room temperature, plates were incubated with peroxidase-conjugated Streptavidin (Jackson ImmunoResearch Laboratories) for 60 minutes at room temperature. Signal was detected with horseradish peroxidase substrate (680) (LI-COR) or with BioFX® One Component horseradish peroxidase Microwell Substrate (SurModics). The ELISA results were normalized to total protein concentration determined by bicinchoninic acid assay (Thermo Scientific) in each plasma sample before the correlation analysis.

2.5. Pull-down assay

Pull-down assay with Dynabeads™ M-280 Streptavidin (Invitrogen) was performed according to the manufacturer's instructions. Details are in Supplementary Methods.

2.6. Human plasma samples

Experiments with plasma from human donors were approved by The Rockefeller University Institutional Review Board. Blood was drawn from two healthy donors (one 26-year-old male and one 36-year-old male), who giving informed written consent, using 21-gauge 0.75-inch butterfly needles into BD Vacutainer™ (BD) 10-mL plastic tubes with sodium heparin (158 USP Units) at The Rockefeller University Hospital. To obtain platelet-poor plasma, blood was centrifuged twice at 2000× g for 10 minutes at room temperature. Platelet-poor plasma was frozen immediately at −80°C.

Plasma was incubated with or without Aβ42 for 60 minutes at 37°C. Aβ42 (Anaspec) was prepared as described previously [14]. Briefly, Aβ42 was suspended in a minimum amount of 1% NH4OH and then diluted to 1 mg/mL in PBS. The concentration of Aβ42 was determined by bicinchoninic acid assay (Thermo Scientific), and the aggregation state was confirmed by transmission electron microscopy at Rockefeller's Electron Microscopy Resource Center.

Plasma from AD patients and ND controls was obtained from University of Kentucky Sanders-Brown Center on Aging (Supplementary Table S1). Blood was drawn into heparinized plastic vacutainer tubes. AD cases were defined by both a clinical diagnosis of AD and a Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuritic

plaque score [16] of B or C, corresponding to probable or definite AD, respectively. ND cases had no clinical diagnosis of AD and a CERAD score of 0. Clinical diagnosis of dementia was established by Mini Mental State Examination (MMSE; score 30 = no dementia and 0 = severe dementia) [17] and Clinical Dementia Rating (CDR; score 0 = no dementia and 3 = severe dementia) [18] scores. AD and ND cases were sex matched and age matched. The protein concentration in each plasma sample was measured with Pierce Bicinchoninic Acid Protein Assay Kit (Thermo scientific) to normalize the ELISA values before correlation analysis. The Western blot data using anti-HK antibody (Abcam) were normalized with transferrin (ab82411; Abcam) loading control as in the study by Zamolodchikov et al. [14].

2.7. Statistical analysis

All analyses were performed using the GraphPad Prism 5 software. Details are in Supplementary Methods.

3. Results

3.1. Generation and screening of monoclonal antibodies specific for HKi/c, HKi, and HKc

Our strategy to detect changes in HKi or HKc levels in human plasma via sandwich ELISA required capture antibodies capable of recognizing both HKi and HKc (HKi/c), as well as detection antibodies specific for HKi or HKc. To generate HKi/c capture antibodies, we immunized Armenian hamsters with peptides derived from the C-terminal end of HK (IQSDDDWIPDIQIDPNGLSC, present in both HKi and HKc), which is part of the HK light chain. To generate HKi- or HKc-specific antibodies, which we expected to be conformational, we immunized hamsters with both purified human HKi and HKc (kallikrein-cleaved human HK; complete cleavage of HK was confirmed by Western blot) (Fig. 1A). In addition, we immunized mice with purified human HKc only. We screened hybridomas generated from immunized animals using direct ELISA with a fluorescent substrate, with plates coated with purified human HKi or HKc proteins (Fig. 1A). After screening more than 6000 wells, we identified a monoclonal antibody recognizing both HKi/c and monoclonal antibodies specific for HKi or HKc. The HKi/c antibody, 3E8, was identified in hybridoma fusions from a hamster immunized with unique light chain peptide (IQSDDDWIPDIQIDPNGLSC) of HK (this sequence is not present in low-molecular-weight kininogen). The HKi-specific antibody, 2B7, was identified in hybridoma fusions from a hamster immunized with HKi and HKc proteins, and the HKc-specific antibody, 4B12, was identified from a mouse immunized with HKc protein.

To determine the specificity of 3E8, 2B7, and 4B12 for HKi and HKc in a purified protein system, we performed the direct ELISAs with the fluorescent substrate using purified antibodies (0.004–3 µg/mL). Antibody 3E8 bound to both HKi/c (Fig. 1B; the signal intensity ratio of HKi to HKc in

3E8 at 0.3 µg/mL = 1.0), whereas antibody 2B7 showed specificity to HKi (Fig. 1C; the ratio of HKi to HKc in 2B7 at 0.3 µg/mL = 10.3), and antibody 4B12 specifically bound to HKc (Fig. 1D; the ratio of HKc to HKi in 4B12 at 0.3 µg/mL = 11.2), in a concentration-dependent manner.

3.2. Development of sandwich ELISAs using HKi/c (3E8), HKi (2B7), and HKc (4B12) antibodies to discriminate between HKi and HKc

To establish if sandwich ELISA is capable of discriminating between HKi and HKc, we tested whether 3E8 could be used as a capture antibody when paired with 2B7 or 4B12 as detection antibodies for detection of HKi and HKc, respectively. Plates were coated with 3E8 capture antibody and then incubated with HKi and HKc proteins mixed in different ratios, then with 2B7 or 4B12 detection antibodies. As shown in Fig. 2A, 2B7 and 4B12 detected HKi and HKc (0–50 ng/well; 0%, 25%, 50%, 75%, and 100% of HKi or HKc), respectively (the signal intensity ratio of 2B7 to 4B12 under the condition of 100% HKi = 7.5; the ratio of 4B12 to 2B7 under 100% HKc = 13.9). This result indicates that the sandwich ELISAs using 3E8, 2B7, and 4B12 can be used as a tool to discriminate between HKi and HKc. To confirm the specificity of each antibody for HKi/HKc versus HKi versus HKc, we performed a pull-down assay testing the ability of all three antibodies to bind HKi/c, HKi, and HKc in solution (Fig. 2B). Western blot analyses using a commercially available anti-HK antibody (Abcam) showed that biotinylated 3E8, 2B7, and 4B12 antibodies can specifically bind to HKi/c, HKi, and HKc, respectively. Biotinylated anti-Aβ42 antibody (control) could not pull down any HKi or HKc protein.

3.3. Detection of HKi- and HKc-level changes in Aβ42-induced contact system activation in normal human plasma by HKi and HKc sandwich ELISAs

Because HKi cleavage is induced in Aβ42-injected mice and Aβ42-treated human plasma [11,14], we next investigated whether the sandwich ELISAs can detect changes in HKi and HKc levels in human plasma after incubation with Aβ42. Western blot using a commercially available anti-HK antibody (Abcam) showed that Aβ42 treatment induced HKi cleavage (as seen through disappearance of the 120-kDa band and the appearance of HKc bands at 45 and 55 kDa) in plasma from two healthy donors (Fig. 3A). When the same samples were analyzed by sandwich ELISA, HKi levels were decreased in Aβ42-activated plasma from both the donors (Fig. 3B; donor 1: 134.3 ± 5.9 vs. 1.1 ± 3.2, P = .0006; donor 2: 107.5 ± 13.7 vs. 2.9 ± 8.3, P = .0026) accompanied by an increase in HKc levels (Fig. 3C; donor 1: 9.7 ± 8.6 vs. 68.9 ± 4.0, P = .004; donor 2: 4.6 ± 7.3 vs. 98.6 ± 5.2, P = .0008) compared with those in nonactivated plasma. These data indicate that Aβ42-induced contact system
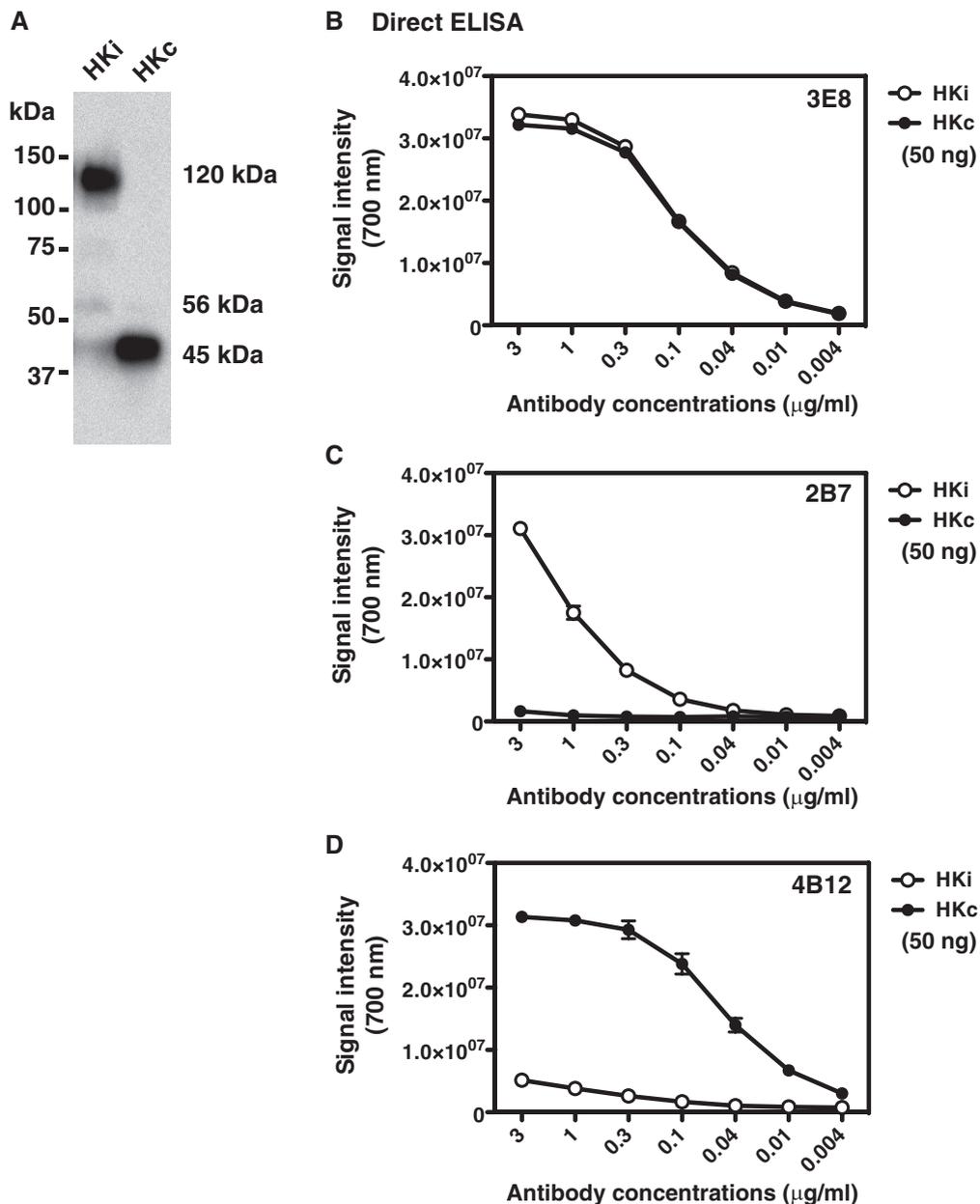
Fig. 1. Identification of monoclonal antibody specific for HKi/c (3E8), HKi (2B7), and HKc (4B12). (A) Western blot using commercially available anti-HK antibody (Abcam) under reducing conditions shows purified human HKi and HKc used for animal immunization and antibody screening. HKi is observed at 120 kDa and the HKc light chain fragment at 45 kDa. Representative Western blot is from three independent experiments. The direct ELISA with a fluorescent substrate using purified (B) 3E8 (HKi/c), (C) 2B7 (HKi), and (D) 4B12 (HKc) antibody (0.004–3 μg/mL). HKi and HKc (50 ng/well) were coated on the plate. The antibodies 3E8, 2B7, and 4B12 showed specificity for HKi/c, HKi, and HKc, respectively. Values are presented as mean ± SD of experiments performed in triplicate. Abbreviations: HKi, intact high-molecular-weight kininogen; HKc, cleaved high-molecular-weight kininogen; SD, standard deviation.

activation and HK cleavage in human plasma can be quantified by the HKi and HKc sandwich ELISAs.

3.4. HKi and HKc ELISAs revealed that changes in HKi and HKc levels in AD patient plasma correlate with cognitive impairment and neuritic plaque pathology

To validate the ELISAs' ability to differentiate between HKi and HKc levels in plasma from AD patients and ND control individuals (as shown previously by Western blot [14]) and to demonstrate their ability to quantitatively measure plasma HKc levels in the same plasma, the HKi and HKc ELISAs were performed on plasma from 10 AD patients and nine ND controls from the University of Kentucky Sanders-Brown Center on Aging (AD patient and control characteristics in Supplementary Table S1). HKi levels were significantly decreased ($P = .0004$; Fig. 4A) accompanied by an increase of HKc ($P = .0003$; Fig. 4B) in AD patients compared with those in ND controls. ELISA and Western blot results were normalized to total protein
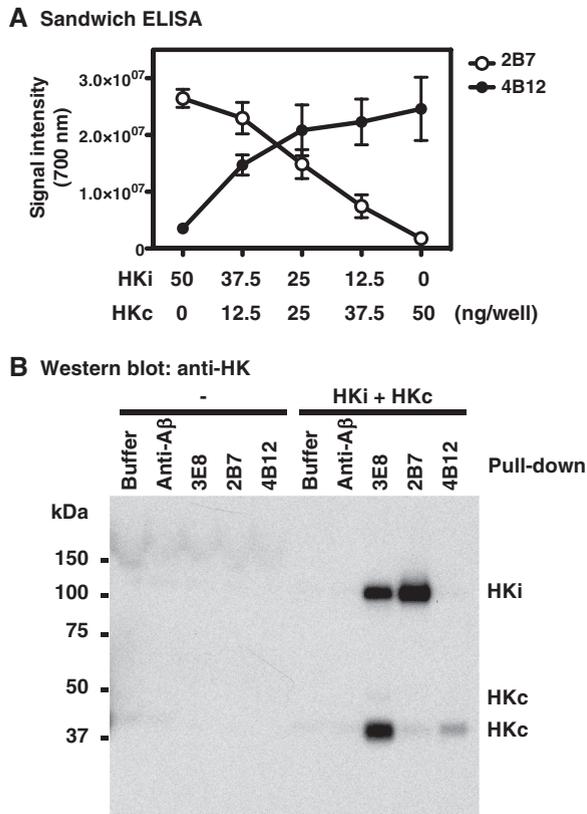
Fig. 2. HKi (2B7)- and HKc (4B12)-specific antibodies are able to discriminate between HKi and HKc in sandwich ELISAs and pull-down assay. (A) Sandwich ELISA with a fluorescent substrate coated with capture antibody 3E8. Plates were incubated with various ratios of HKi and HKc protein as indicated on the x-axis (0–50 ng/well). 2B7 and 4B12 can discriminate between HKi and HKc under the mixed condition of HKi/c in the sandwich ELISAs. Values are presented as mean ± SD from three independent experiments. (B) Pull-down assay with streptavidin beads. Biotinylated 3E8, 2B7, and 4B12 antibodies were incubated with or without HKi and HKc protein and then pulled down with streptavidin beads. Western blot analysis in representative samples was performed using commercial anti-HK antibody (Abcam), showing the selective binding of 3E8, 2B7, and 4B12 for HKi/c, HKi, and HKc, respectively. HKi and HKc proteins used in the assay (input, 125 ng each/lane) were shown in Fig. 1A (100 ng each/lane). Anti-Aβ antibody was used as a negative control. Replicates are biological replicates (n = 3). Abbreviations: HKi, intact high-molecular-weight kininogen; HKc, cleaved high-molecular-weight kininogen; Aβ, β-amyloid; SD, standard deviation.

concentration and transferrin, respectively, and results were compared between the two assays. There was a significant positive correlation between HKi levels obtained for these samples by Western blot [14] and those by ELISA (Fig. 4C; $r = 0.86$; $P < .0001$). Furthermore, HKc levels obtained by ELISA were inversely correlated with HKi levels obtained by Western blot [14] (Fig. 4D; $r = -0.88$, $P < .0001$), indicating that the two assays are internally consistent and that the HK-level results for this sample set are reproducible over time.

To determine whether contact system activation in AD correlates with established measures of disease progression, we analyzed the relationship between HKi/c values and two

dementia scores, MMSE [17] and CDR [18], as well as the postmortem CERAD neuritic plaque score [16]. MMSE scores for cognitively normal individuals range from 25 to 30, with 20–24 considered mild dementia, 13–20 moderate dementia, and <12 severe dementia. Lower MMSE scores (cognitive dysfunction) were found in individuals with increased HK cleavage, as indicated by positive correlation with HKi levels and inverse correlation with HKc levels (Fig. 5A and B; HKi: $r = 0.52$, $P = .022$; HKc: $r = -0.62$, $P = .005$). CDR scores range from 0 (normal) to 3 (severe dementia). Similar to MMSE scores, CDR scores associated with cognitive dysfunction correlated with increased HK cleavage (Fig. 5C and D; HKi: $r = -0.57$, $P = .011$; HKc: $r = 0.65$, $P = .003$). CERAD scores are based on Aβ plaque pathology during brain autopsy, where 0 = normal, B = probable AD, and C = definite AD. A significant correlation was found between CERAD score and HK levels; individuals with increased HKi cleavage, as evidenced by lower HKi levels and higher HKc levels detected by ELISA, had higher CERAD scores (Fig. 5E and F; HKi: $r = -0.62$, $P = .005$; HKc: $r = 0.81$, $P < .0001$).

4. Discussion

Here, we developed novel sandwich ELISAs that can discriminate between HKi and HKc in human plasma. Because cleavage of HKi by plasma kallikrein causes dramatic conformational changes in the protein [19], we expected that finding antibodies that can distinguish HKc from HKi would be easy. Surprisingly, we had to screen over 6000 hybridomas before finding an HKc-specific antibody (4B12), whereas HKi/c (3E8) and HKi (2B7) antibodies were identified more quickly. Although the reasons behind the rarity of HKc-specific antibodies are not clear, one possibility is that the HKc-specific conformation is not highly immunogenic. The epitope of the capture antibody 3E8 is within the C-terminus of the HK light chain (IQSDDDWIPDIQIDPNGLSC), which does not block the epitopes of HKi and HKc recognized by 2B7 and 4B12, respectively. In the pull-down assay, it appeared that 4B12 does not pull down HKc as readily as 3E8 does, indicating that it might be more difficult for 4B12 to bind to HKc in solution than to HKc captured with 3E8 on the plate in the sandwich ELISA and HKc on the plate in the direct ELISA.

AD pathology begins years, even decades, before the onset of cognitive dysfunction [20]. One of the major impediments to AD treatment is insufficient early diagnosis. AD biomarkers that are capable of providing early indication of AD onset exist (like cerebral spinal fluid levels of Aβ42 and tau and brain amyloid imaging), but they are invasive, costly, and time-consuming [20,21]. To address this need, blood biomarkers have been sought for many years. Our study suggests that plasma levels of HKi/c as measured by ELISA may be useful as an easily accessible biomarker for AD. Because plasma HKi cleavage shows positive
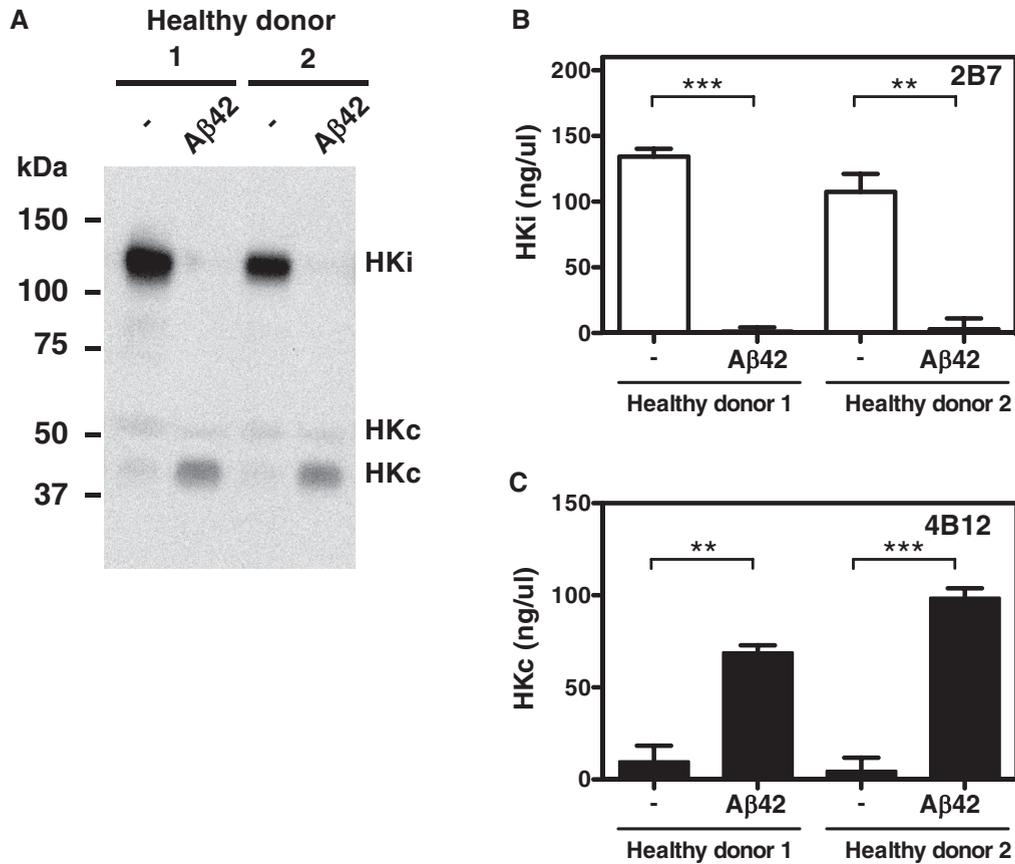
Fig. 3. Aβ42-induced contact system activation and HK cleavage in human plasma can be quantified by sandwich ELISAs. Plasma from healthy donors 1 and 2 was incubated with buffer (0.08% NH₄OH/PBS) or Aβ42 (20 μM) for 60 minutes at 37°C. (A) Western blot using anti-HK antibody (Abcam) showed that Aβ42 induced HKi cleavage in both donors' plasma. Representative Western blot from three independent experiments. (B) HKi and (C) HKc sandwich ELISAs with 1:3000 sample dilution. In both donors, HKi levels were decreased in Aβ42-activated plasma (donor 1: 134.3 ± 5.9 vs. 1.1 ± 3.2, $P = .0006$; donor 2: 107.5 ± 13.7 vs. 2.9 ± 8.3, $P = .0026$) accompanied by the increase of HKc levels (donor 1: 9.7 ± 8.6 vs. 68.9 ± 4.0, $P = .004$; donor 2: 4.6 ± 7.3 vs. 98.6 ± 5.2, $P = .0008$) compared with those in vehicle-treated plasma. The change in HK levels detected in the ELISAs was consistent with the Western blot profiles. Values are presented as mean ± SD from three independent experiments. Comparisons between groups were performed using the two-tailed paired t-test. **$P < .01$; ***$P < .001$. Abbreviations: HKi, intact high-molecular-weight kininogen; HKc, cleaved high-molecular-weight kininogen; Aβ, β-amyloid; SD, standard deviation.

correlation with cerebral spinal fluid Aβ42 [14], an early disease biomarker, and plasma Aβ42 levels are elevated in the early pathogenesis of AD [21,22], which could contribute to the inflammatory and thrombotic pathologies through the activation of FXII-induced contact system [10–15], our HKi/c ELISAs may be useful in detecting and monitoring early stages of AD development and progression. Because contact system activation is not unique to AD, it might be necessary to determine HKi/c values discriminating AD from other diseases related to contact system activation [2–9,23].

Because the CERAD neuritic plaque score is used for postmortem diagnosis for AD [16], we should note that when we analyze the relationship between plasma HKi/c values and CERAD score, the date of blood draw relative to the date of death may be different among individuals (days from the date of blood draw to the date of death in CE-RAD score 0 = 853.4 ± 761.4, B = 1661.0 ± 1137.2, and C = 747.9 ± 545.9). Although there were no significant dif-

ferences in the days among the scores, there was a trend toward a longer interval between date of blood draw and date of death in CERAD score B cases. However, even when CE-RAD score B cases were excluded, there was still a significant correlation between HKi/c values and CERAD score (HKi: $r = -0.66$, $P = .006$; HKc: $r = 0.83$, $P < .0001$).

Interestingly, when we measured the HKi/c levels in the plasma of 10 AD patients, two AD cases did not have more HKc than HKi, consistent with the less FXII activation determined by Western blot [14]. One explanation of lower HKi cleavage in some AD cases is that AD is a complex, heterogeneous disease that likely has different driving mechanisms for different patients [20,24]. It is possible that disease in these individuals is driven by a different mechanism, one that does not involve FXII activation or has FXII activation as a by-product. On the other hand, an ND plasma had more HKc than HKi consistent with FXII activation [14]. The comorbidities of diabetes, hypertension, and hypercholesterolemia present in an ND case might affect
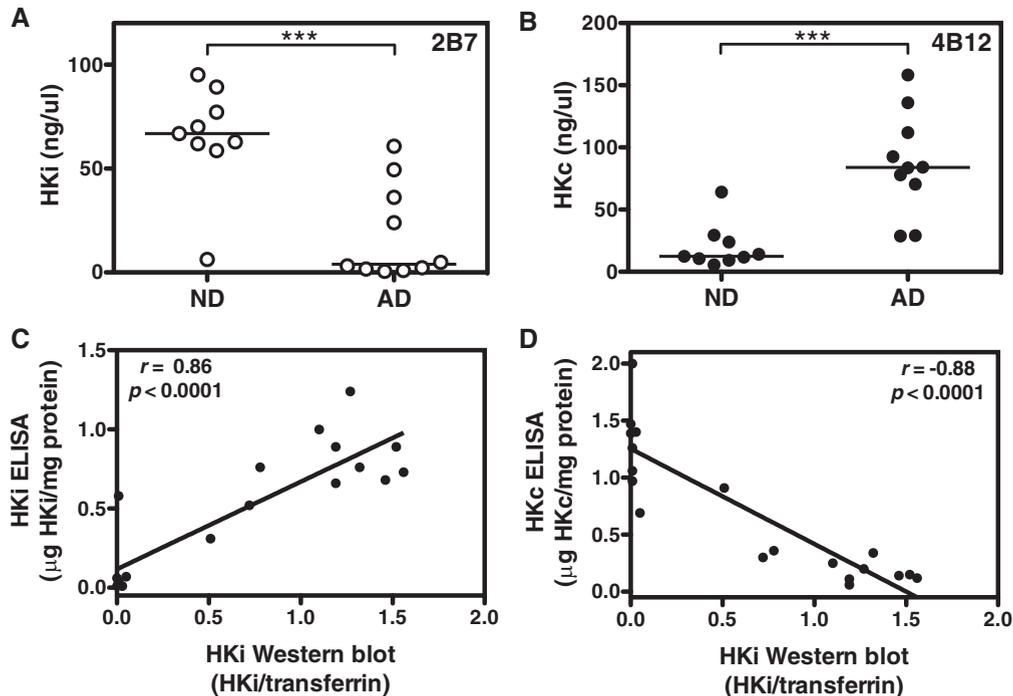
Fig. 4. Sandwich ELISAs revealed changes in HKi and HKc levels in AD patient plasma and a significant correlation to HKi and HKc levels obtained for these samples by Western blot. (A and B) The HKi and HKc ELISAs were performed with 1:3000 sample dilution in human plasma from 9 ND controls and 10 AD patients from University of Kentucky Sanders-Brown Center on Aging. HKc level was significantly increased ($P = .0003$), accompanied by the decrease of HKi level in AD patients ($P = .0004$), compared with that in ND controls. Data are presented as vertical scatter plots with medians, and experiments were performed in triplicate. Comparison between groups was performed using the two-tailed Mann-Whitney test. *** $P \leq .001$. Each value in these individuals detected in the ELISAs is shown in Supplementary Fig. S1. (C and D) Correlation between ELISA values and Western blot profiles in HKi and HKc. The HKi and HKc ELISA values were normalized with the protein concentration in each plasma sample, and the Western blot profiles of HKi were normalized to transferrin [14]. There were significant correlations between ELISA values and Western blot profiles in HKi ($r = 0.86$, $P < .0001$) and HKc ($r = -0.88$, $P < .0001$). Correlations were examined using Pearson's correlation coefficient (r). Abbreviations: AD, Alzheimer's disease; HKi, intact high-molecular-weight kininogen; HKc, cleaved high-molecular-weight kininogen; ND, nondemented.

FXII activation because some of the other factors can activate the contact system in thromboinflammatory diseases [2,3], autoimmune diseases [4], and hyperlipidemia [5].

More importantly, optimal blood collection protocols for the ELISAs remain to be determined because there might be differences in *ex vivo* contact activation between blood drawn into heparinized plastic vacutainer tubes and EDTA-coated syringes as described previously [14]. The AD and control patient plasma samples used in this study were prepared from blood collected in heparinized vacutainer tubes via vacuum. When we measured HKi and HKc levels in another cohort where the blood was drawn into EDTA-coated syringes via aspiration (ND: n = 10 [aged 70.5 ± 4.0 years], CDR score = 0 ± 0; AD: n = 10 [aged 73.6 ± 5.8 years], CDR score = 1.0 ± 0.58), HKi levels were significantly decreased in AD plasma ($P = .015$ analyzed by two-tailed Mann-Whitney test), and there was a trend toward higher HKc levels ($P = .166$ analyzed by two-tailed Mann-Whitney test), compared with those in ND controls. We have observed that in the enzyme-substrate reaction step, HKc ELISAs run on plasma samples collected into EDTA-coated syringes developed slower (over 8 minutes) than HKc ELISAs run on plasma samples collected in hepa-

rinized tubes (approximately 3 minutes), suggesting that more HKc, which can be detected by ELISA, is present in the samples prepared in heparin-coated tubes. One possible explanation is that heparin binding to HKc [25] could induce a conformational change in HKc, allowing it to be more easily recognized by 4B12, and/or the presence of EDTA could not induce the conformational change in HKc necessary for optimal recognition by 4B12 [26]. Therefore, the plasma sample preparation method needs to be further optimized for HKi and HKc ELISA analyses.

Increased HKc and bradykinin could play crucial roles in the development of AD pathologies including vascular abnormalities [27] and neuroinflammation [28]. The biological activity of bradykinin mostly affects vascular function and inflammatory processes such as vasodilatation, increase in vascular permeability, and cell recruitment to the site of inflammation [29]. Of note, the blockade of bradykinin B1 receptor suppresses Aβ deposition and neuroinflammation in a mouse model of AD [30], supporting a role for bradykinin signaling in AD pathogenesis. Although HKc function in AD etiology has not been studied in detail, recent reports implicate HKc in various processes: (1) inhibition of endothelial proliferation and suppression of neovascularization
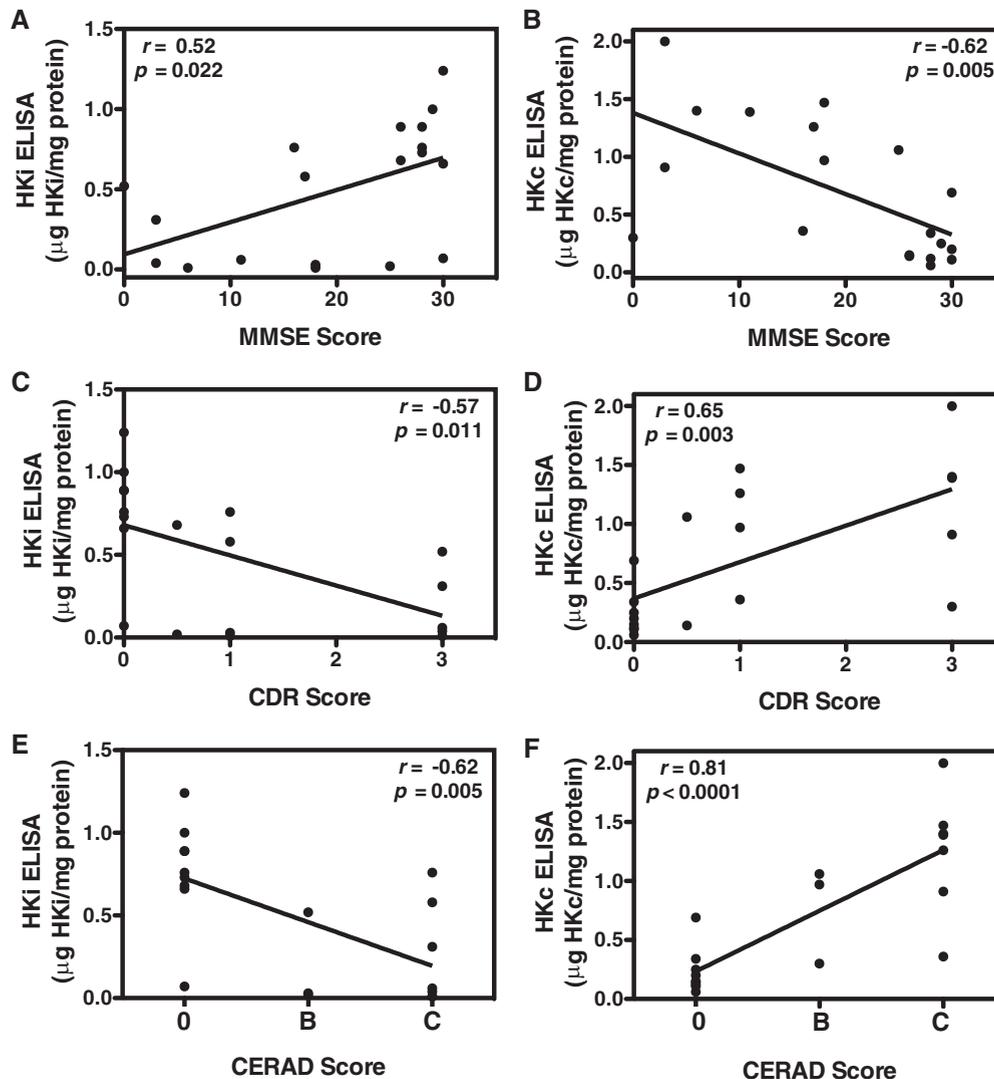
Fig. 5. Sandwich ELISAs demonstrate that HKi- and HKc-level changes in AD patient plasma correlate with dementia and neuritic plaque scores. The HKi (A, C, and E) and HKc (B, D, and F) ELISAs were performed with 1:3000 sample dilution and then normalized with the protein concentration in each plasma of 9 ND controls and 10 AD patients from University of Kentucky Sanders-Brown Center on Aging. For dementia scores, MMSE (A and B), for which 30 = no dementia and 0 = severe dementia, and CDR (C and D), for which 0 = no dementia and 3 = severe dementia, were used. (E and F) Postmortem CERAD neuritic plaque score of 0 corresponds with ND, (B) corresponds with probable AD, and (C) corresponds with definite AD. HKi and HKc level changes as detected by the ELISAs were significantly correlated with MMSE (HKi: $r = 0.52$, $P = .022$; HKc: $r = -0.62$, $P = .005$), CDR scores (HKi: $r = -0.57$, $P = .011$; HKc: $r = 0.65$, $P = .003$), and CERAD (HKi: $r = -0.62$, $P = .005$; HKc: $r = 0.81$, $P < .0001$). Correlations were analyzed using Pearson's correlation coefficient (r). Abbreviations: CERAD, Consortium to Establish a Registry for Alzheimer's Disease; MMSE, Mini Mental State Examination; CDR, Clinical Dementia Rating; AD, Alzheimer's disease; HKi, intact high-molecular-weight kininogen; HKc, cleaved high-molecular-weight kininogen; ND, nondemented.

of subcutaneously implanted matrigel plugs [31,32]; (2) inhibition of neointima formation after vascular injury [33]; (3) acceleration of the onset of endothelial progenitor cell senescence by activating reactive oxygen species [34]; and (4) inhibition of Mac-1–dependent leukocyte adhesion [35]. These reports suggest that HKc could be involved in the unregulated vascular function and inflammatory process in AD pathogenesis.

It has been reported that HKc detection in hereditary angioedema patients with 1:64 sample dilution is enhanced by the addition of dextran sulfate (DXS) to the ELISA [23]. We investigated whether DXS could enhance the signal in our ELISA system and found that DXS enhanced the signals both of HKi and HKc ELISAs in the presence of proteinase inhibitor (not shown). Because our ELISA is much more sensitive than the system previously described, allowing measurement of plasma HKi and HKc levels with much higher sample dilution (1:3000 to 1:5000), we needed to dilute samples even more to use DXS as an enhancer.

In conclusion, we developed two sandwich ELISAs for measuring HKi and HKc levels in human plasma using three types of monoclonal antibodies. The ELISAs can be useful as a simple and highly sensitive diagnostic tool for AD and other diseases that show excessive HKi cleavage and bradykinin release, as well as research tools for investigating mechanisms related to contact system activation.

Acknowledgments

Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.dadm.2018.06.008.

RESEARCH IN CONTEXT

1. Systematic reviews: A literature review showed that contact system activation is increased in the plasma of Alzheimer's disease (AD) patients and mouse models. Contact activation can be measured by monitoring high-molecular-weight kininogen (HK) cleavage. HK cleavage is typically detected by Western blot, but for potential diagnostic purposes, a more quantitative method to measure plasma HKc levels with high sensitivity and specificity is needed.

2. Interpretations: We generated three monoclonal antibodies specifically recognizing human HKi/c, HKi, or HKc and developed two sandwich ELISAs. The HKi/HKc changes in AD plasma as revealed by the ELISAs were significantly correlated with dementia and neuritic plaque scores, suggesting these ELISAs can be an easily accessible diagnostic tool for AD.

3. Future directions: These ELISAs will need further validation with larger sample sets. Furthermore, a longitudinal assay is needed to determine the timeline of plasma HK cleavage in AD. It will also be necessary to determine HKi/c values discriminating AD from other diseases related to contact system activation.

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