Anti-HK antibody reveals critical roles of a 20-residue HK region for Aβ-induced plasma contact system activation

Tracking no: ADV-2021-006612R2

Zu-Lin Chen (The Rockefeller University, United States) PRADEEP SINGH (THE ROCKEFELLER UNIVERSITY, United States) Katharina Horn (Rockefeller University, United States) Sidney Strickland (The Rockefeller University, United States) Erin Norris (Rockefeller University, United States)

Abstract:
Alzheimer's disease (AD) is a neurodegenerative disorder and the leading cause of dementia. Vascular abnormalities and neuroinflammation play roles in AD pathogenesis. Plasma contact activation, which leads to fibrin clot formation and bradykinin release, is elevated in many AD patients, likely due to the ability of AD's pathogenic peptide β-amyloid (Aβ) to induce its activation. Since overactivation of this system may be deleterious to AD patients, the development of inhibitors could be beneficial. Here, we show that 3E8, an antibody against a 20-amino acid region of high molecular weight kininogen's (HK) domain 6, inhibits Aβ-induced intrinsic coagulation. Mechanistically, 3E8 inhibits contact system activation by blocking the binding of prekallikrein (PK) and factor XI (FXI) to HK, thereby preventing their activation and the continued activation of factor XII (FXII). The 3E8 antibody can also disassemble HK/PK and HK/FXI complexes in normal human plasma in the absence of a contact system activator due to its strong binding affinity for HK, indicating its prophylactic ability. Furthermore, the binding of Aβ to both FXII and HK is critical for Aβ-mediated contact system activation. These results suggest that a 20-amino acid region of HK's domain 6 plays a critical role in Aβ-induced contact system activation, and this region may provide an effective strategy to inhibit or prevent contact system activation in related disorders.

Conflict of interest: COI declared - see note

COI notes: The 3E8, 2B7, and 4B12 anti-HK antibodies have been licensed to Millipore Sigma.

Preprint server: No;

Author contributions and disclosures: Z.L.C. designed the study, performed experiments, analyzed data, and wrote the manuscript; P.S. and K.H. performed experiments and analyzed data; S.S. helped design the study and analyze data; E.H.N. designed the study, participated in data analysis, and wrote the manuscript.

Non-author contributions and disclosures: No;

Agreement to Share Publication-Related Data and Data Sharing Statement: For original data, please contact the corresponding author at enorris@rockefeller.edu.

Clinical trial registration information (if any):
Anti-HK antibody reveals critical roles of a 20-residue HK region for Aβ-induced plasma contact system activation

Running title: Mechanisms of an HK cleavage-blocking antibody

Zu-Lin Chen, Pradeep Kumar Singh¹, Katharina Horn¹, Sidney Strickland, and Erin H. Norris*

The Rockefeller University
Patricia and John Rosenwald Laboratory of Neurobiology and Genetics
New York, NY 10065, USA

¹These authors contributed equally to this paper.

*Corresponding author: Erin H. Norris, The Rockefeller University, Email: enorris@rockefeller.edu.

Data sharing statement: For original data, please contact the corresponding author at enorris@rockefeller.edu.

Category: Thrombosis and Hemostasis

Title characters: 113

Running title characters: 47

Key point: 2 (106 & 120 characters)

Abstract word counts: 218
Text word counts: 3980

Figures and tables: 7

References: 74

**Key points:**

1. A 20-residue region in domain 6 of HK plays a critical role in Aβ42-induced contact system activation.
2. 3E8 also blocks PK and FXI binding to HK, disassembles HK/PK and HK/FXI complexes, and delays intrinsic coagulation.

**Keywords:** High molecular weight kininogen (HK), Alzheimer's disease (AD), prekallikrein (PK), factor XII (FXII), factor XI (FXI), β-amyloid (Aβ), activated partial thromboplastin time (aPTT), prothrombin time (PT).

**Abstract**

Alzheimer's disease (AD) is a neurodegenerative disorder and the leading cause of dementia. Vascular abnormalities and neuroinflammation play roles in AD pathogenesis. Plasma contact activation, which leads to fibrin clot formation and bradykinin release, is elevated in many AD patients, likely due to the ability of AD's pathogenic peptide β-amyloid (Aβ) to induce its activation. Since overactivation of this system may be deleterious to AD patients, the development of inhibitors could be beneficial. Here, we show that 3E8, an antibody against a 20-amino acid region of high molecular weight kininogen's (HK) domain 6, inhibits Aβ-induced intrinsic coagulation.
Mechanistically, 3E8 inhibits contact system activation by blocking the binding of prekallikrein (PK) and factor XI (FXI) to HK, thereby preventing their activation and the continued activation of factor XII (FXII). The 3E8 antibody can also disassemble HK/PK and HK/FXI complexes in normal human plasma in the absence of a contact system activator due to its strong binding affinity for HK, indicating its prophylactic ability. Furthermore, the binding of Aβ to both FXII and HK is critical for Aβ-mediated contact system activation. These results suggest that a 20-amino acid region of HK’s domain 6 plays a critical role in Aβ-induced contact system activation, and this region may provide an effective strategy to inhibit or prevent contact system activation in related disorders.
Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder and a major cause of dementia. The pathological hallmarks of AD are β-amyloid (Aβ) plaques, tau tangles, neuroinflammation, and neuronal degeneration. There is evidence that vascular dysfunction is also a core pathology of AD\(^1\)\(^-\)\(^3\). Not only is disruption of the blood-brain barrier (BBB) an early biomarker of cognitive dysfunction\(^4\), but cerebrovascular and blood flow abnormalities are also associated with cognitive decline and AD progression\(^5\)\(^-\)\(^7\). AD is a complex and heterogeneous disease, and it is unlikely that only one mechanism can be targeted for treatment of all patients\(^8\). It is therefore important to identify various pathogenic pathways which will allow stratification of patients and personalized treatment.

Dysfunctional blood clotting is often observed in AD patients and mouse models\(^7\)\(^,\)\(^9\)\(^-\)\(^18\). Parenchymal brain deposits of fibrin, a proinflammatory protein that is also the main component of blood clots\(^19\)\(^,\)\(^20\), play a significant role in AD progression in mouse models\(^9\)\(^,\)\(^10\)\(^,\)\(^19\)\(^-\)\(^24\). Fibrin and its precursor, fibrinogen, are blood proteins, and their presence in the brain indicates a loss of BBB integrity in these patients. Fibrin is formed upon generation of thrombin during the clotting pathway of the contact activation system.

The plasma contact system, which includes both clotting and inflammatory pathways\(^25\), may play a role in AD pathogenesis\(^14\)\(^,\)\(^26\). Aβ\(^42\), one of the primary pathogenic factors of AD\(^27\)\(^-\)\(^29\), can activate factor XII (FXIIa), to initiate the contact system\(^30\)\(^-\)\(^33\). FXIIa activation of factor XI (FXI) triggers the intrinsic clotting pathway, leading to thrombin generation.
and fibrin formation\textsuperscript{32,34}, whereas FXIIa activation of prekallikrein (PK) leads to the release of bradykinin from its precursor high molecular weight kininogen (HK) and subsequent activation of inflammatory processes\textsuperscript{6,32,35-39}. Both fibrin formation and bradykinin-mediated inflammation may contribute to AD\textsuperscript{9,18,22}. It has be shown that the AD brain parenchyma exhibits higher plasma kallikrein (PK\textsubscript{a}) activity\textsuperscript{40}, and Aβ plaques contain FXII\textsuperscript{41} and bradykinin\textsuperscript{18}. Compared to non-demented individuals, AD patients have increased levels of cleaved HK (cHK) in their cerebrospinal fluid (CSF)\textsuperscript{42} and higher plasma levels of FXIIa, cHK, and bradykinin\textsuperscript{15,18,43}. In a mouse model of AD, plasma contact system activation is also elevated\textsuperscript{43}, and knockdown of this system using an anti-FXII antisense oligonucleotide (ASO) ameliorates AD pathology and cognitive impairment in a mouse model\textsuperscript{35}. Therefore, inhibition of the contact system could be beneficial for AD patients.

We previously showed that an anti-HK antibody (clone 3E8, raised against a 20-amino acid region of HK’s domain 6) blocks Aβ42-induced HK cleavage and bradykinin release\textsuperscript{36}. Our new studies revealed that 3E8 also prevents Aβ42-induced intrinsic coagulation. Mechanistically, 3E8 inhibits contact system activation by blocking the binding of PK and FXI to HK, thereby preventing activation of PK and FXI and continued activation of FXII. We also show here that 3E8 disassembles HK/PK and HK/FXI complexes in normal human plasma in the absence of a contact system activator, suggesting its prophylactic ability. Moreover, our results revealed that Aβ42 can bind HK, which is critical for Aβ42-mediated contact system activation. Therefore, our results show for the first time that a 20 amino acid region of HK plays an essential role in Aβ42-
mediated contact system activation, and targeting this region may provide an effective strategy to treat contact system-related pathological conditions such as AD.

**Materials and Methods**

**Preparation of anti-HK antibodies and Aβ42**

3E8, 2B7, and 4B12 anti-HK antibodies\(^{44}\) and Aβ42\(^{43,44}\) (Anaspec) were prepared as previously described. The aggregation state of Aβ42 was confirmed by transmission electron microscopy at Rockefeller’s Electron Microscopy Resource Center.

**Binding Experiments**

The binding experiments were performed using ELISA\(^{45}\). For 3E8 binding to HK, plates were coated with 2 μg/ml 3E8 in binding buffer (0.1 M sodium bicarbonate, pH 9.6). For PK and FXI binding to HK, plates were coated with 2 μg/ml of PK or FXI in binding buffer, respectively. The plates were then incubated with various concentrations of HK in the binding buffer. Bound HK was detected with antibody to HK as described\(^{44}\). The dissociation constant (K\(_D\)) of HK and 3E8 interaction was determined from the binding curve using a non-linear regression curve fit equation in GraphPad Prism software. For 3E8 blocking, PK/HK binding, or FXI/HK binding studies, HK was preincubated at 37°C in the absence or presence of increasing concentrations of the 3E8 antibody. Bound HK was then determined as described above.

**Blood collection and plasma preparation**
The collection and preparation of human plasma was approved by The Rockefeller University Institutional Review Board. Blood from healthy human donors (n=6) who had given informed, written consent was collected at The Rockefeller University Hospital. Plasma was prepared as described previously\textsuperscript{44}. Pooled normal human plasma (NHP) and FXII-deficient human plasma (FXII-DF) were purchased from George King Bio-Medicals, and kininogen-deficient human plasma (KN-DF) was purchased from Technoclone.

**\textbf{Aβ42 pulldown experiments and anti-HK antibody immunoprecipitation}**

NHP, FXII-DF, or KN-DF human plasma was incubated with B-Aβ42 in the presence or absence of 3E8 anti-HK antibody. Dynabeads\textsuperscript{TM} M-280 Streptavidin (Invitrogen) was used to pull down the B-Aβ42/bound proteins complex according to the manufacturer’s instructions.

For anti-HK antibody immunoprecipitation, anti-HK antibodies (3E8, 2B7, 4B12)\textsuperscript{44} and control IgG (Innovative Research) were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) according to the manufacturer’s instructions. EDTA-Plasma was incubated with biotinylated HK antibodies and control IgG. In 2B7 anti-HK antibody pulldown experiments, NHP was incubated with 3E8/PBS/IgG, then incubated with B-2B7 at 37°C for 20 min. Dynabeads\textsuperscript{TM} M-280 Streptavidin was used to pull down the antibody-antigen complex. Samples were eluted with sodium dodecyl sulfate (SDS) sample buffer, and Western blots were performed.

**\textbf{HK D6 peptide competition experiments}**
A 20-residue peptide derived from domain 6 of HK (IQSDDDWIPDIQIDPNGLSF) was synthesized at Rockefeller’s Proteomics Resource Center. This peptide (hereafter referred to as HK-D6) was used as an antigen to generate the 3E8 anti-HK antibody. Human plasma was incubated with buffer; 3E8 anti-HK antibody (0.5 μM); control IgG (0.5 μM); HK-D6 (0.5 μM, 1 μM, 2μM); or 3E8 anti-HK antibody or control IgG (0.5 μM) + HK-D6 (0.5 μM, 1 μM, 2 μM). Incubations were left at 37°C for 20 minutes, and then Aβ42 (5 μM) was added and incubated for two hours. Western blotting analyses were performed.

Western blotting

Western blots were performed as described previously46. Briefly plasmas after various treatments were heated to 95°C for 5 minutes in sample buffer. Equal amounts of plasma from each sample were run on SDS polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane (PVDF, Bio-Rad), and analyzed by Western blot with antibodies against FXII/FXIIa (Cedarlane Laboratories), FXI (Haematologic Technologies), PK (Affinity Biological), HK and cHK (3E8), HK light chain (Abcam), low molecular weight kininogen (LMWK, Novus biological), and transferrin (TF) (Abcam). Blots were imaged via Bio-Rad ChemiDoc™. Protein levels were quantified by densitometry with NIH Image J. All experiments used plasmas from 6 healthy donors, were run in duplicate, and repeated at least three times.

Plasma kallikrein activity

EDTA-plasma was incubated with buffer, control IgG (50 nM), or various concentrations of 3E8 anti-HK antibody (5 nM, 25 nM, and 50 nM) at 37°C for 20 minutes in plates.
Aβ42 (5 μM) and chromogenic substrate S-2302 (0.67 mM final concentration, Diapharma) were added, and absorbance at 405 nm was read for 60 min at 37°C in a spectrophotometer (Molecular Devices). Plasmas from six different human donors were used, and each plasma was used twice.

**Activated partial thromboplastin time (aPTT) and prothrombin time (PT) tests in human plasma**

A spectrophotometer-based aPTT assay was performed as described previously\(^\text{15}\) with some modifications. Briefly, citrated-plasma was warmed at 37°C for 3 minutes and incubated with buffer or control IgG (3.75 μM) or various concentrations of 3E8 anti-HK antibody (0.05 μM, 0.15 μM, 0.75 μM, and 3.75 μM) at 37°C for 20 minutes in a 96-well plate. Then aPTT reagent solution (30 μl; APTT-XL; Pacific Hemostasis) was added and incubated for 5 minutes. Clot formation was initiated by adding 30 μl of 25 mM CaCl\(_2\) solution. Clot formation was monitored kinetically at 350 nm at 37°C over time using SoftMax Pro 6.1 software (Molecular Devices) as described previously\(^\text{15}\).

For the effects of 3E8 anti-HK antibody on Aβ42-induced intrinsic coagulation changes, citrated-plasma was incubated with buffer or control IgG (3.75 μM) or various concentrations of 3E8 (0.05 μM, 0.15 μM, 0.75 μM, and 3.75 μM) at 37°C for 20 minutes. Aβ42 (4 μM) was added and incubated at 37°C for 10 minutes, then CaCl\(_2\) solution was added, and clotting was determined as described above.

For PT, citrated-plasma was warmed to 37°C for 3 minutes and incubated with buffer, control IgG, or 3E8 anti-HK antibody (3.75 μM) at 37°C for 20 minutes in 96-well plates.
Clotting was monitored kinetically as described above after addition of PT reagent Thromboplastin D, Pacific hemostasis).

**Statistical analyses**

All statistical analyses were performed using GraphPad Prism 4 software. Comparisons among multiple groups were performed using one-way ANOVA followed by Newman-Keuls multiple comparison test.

**Results**

**Characterization of the 3E8 anti-HK antibody.**

The 3E8 anti-HK antibody dose-dependently blocks Aβ42-induced HK cleavage and bradykinin release in human plasma *ex vivo*[^36^]. To investigate the specificity of this antibody, we performed Western blotting with purified proteins and human plasmas and probed with 3E8 (Fig 1A). 3E8 detected purified human HK and cHK, and it showed a single band corresponding to HK in normal human plasma (NHP) and a single band corresponding to cHK in Aβ42-treated human plasma. There were no bands detected in kininogen-deficient (KN-DF) plasma. When the membrane was stripped and re-probed with an antibody against low molecular weight kininogen (LMWK), LMWK was detected in NHP and in Aβ42-treated human plasma, but not in KN-DF plasma (Fig 1A). This LMWK band was not revealed by 3E8 anti-HK antibody, indicating 3E8 does not recognize LMWK and is specific for HK and cHK.
We also performed immunoprecipitation using 3E8, 2B7 (intact HK-specific), and 4B12 (cHK-specific) antibodies. Both 3E8 and 2B7 immunoprecipitated intact HK, but not FXII or PK, from NHP (Fig 1B), indicating that 3E8 and 2B7 do not bind FXII or PK. Neither 4B12 nor control IgG pulled down intact HK from NHP. This experiment showed that 3E8 binds HK but not other contact system proteins in human plasma. Furthermore, using ELISA we determined that the $K_D$ of 3E8 for HK is 168 ± 38 pM, which indicates very strong binding affinity (Fig 1C).

The inhibitory effects of 3E8 anti-HK antibody on Aβ42-induced HK cleavage are abolished by the competitive HK-D6 peptide.

The 3E8 anti-HK antibody was generated using a peptide derived from domain 6 (D6) of human HK. To analyze whether 3E8 blocks HK cleavage by binding specifically to HK’s D6 in human plasma, we examined 3E8’s inhibitory effects on HK-cleavage in the presence of various concentrations of HK-D6 (Fig 2). Aβ42 induced HK cleavage, and HK cleavage was blocked by 3E8 (Lanes 2 and 3, Fig 2A). Under the same conditions, HK-D6 did not show much effect on Aβ42-induced HK cleavage (Lanes 5-7, Fig 2A). However, HK-D6 dose-dependently blocked the inhibitory effects of 3E8 on Aβ42-induced HK cleavage (Lanes 8-10, Fig 2A). Control IgG and IgG+HK-D6 did not show any effect on Aβ42-induced HK cleavage (Lanes 4 and 11-13, Fig 2A). These results suggest that the inhibitory effect on HK cleavage by 3E8 is dose-dependently abolished by the competitive HK-D6 peptide (Fig 2B), confirming that 3E8 blocks HK cleavage by binding to this 20-amino acid region of HK’s D6 (antigen of 3E8) in human plasma.
3E8 dose-dependently inhibits Aβ42-induced PK and FXII activation

The 3E8 anti-HK antibody binds to HK’s D6, the region that binds PK and FXI and hence plays an important role in contact system activation\(^4^7\)\(^-\)\(^5^0\). We analyzed whether 3E8 impacts PK activation. 3E8 dose-dependently inhibited Aβ42-induced HK cleavage\(^3^6\) as well as PK activation (Fig 3A & 3B). We also analyzed whether 3E8 inhibits Aβ42-induced kallikrein activity using a chromogenic substrate. In the absence of 3E8, Aβ42 induced kallikrein generation in NHP. However, 3E8 dose-dependently inhibited Aβ42-induced kallikrein activation (Fig 3C). Control IgG had no effect on kallikrein activity.

Since activated PK (kallikrein) can feed back to activate FXII\(^5^1\) and 3E8 inhibited PK activation, we investigated whether 3E8 affected FXII activation. We found that 3E8 dose-dependently inhibited FXII activation (Fig 3D & 3E). These results suggest that 3E8 anti-HK antibody dose-dependently prevents both PK and FXII activation. Since our immunoprecipitation analyses showed that 3E8 only binds HK but not FXII or PK in human plasma (Fig 1B), the effects of 3E8 on FXII or PK activation are through its direct binding to HK, not FXII or PK.

3E8 has the same effect on Aβ42-induced FXII activation as does HK deficiency in human plasma.

We compared the effect of 3E8 anti-HK antibody with that of HK deficiency (using KN-DF human plasma) on Aβ42-induced FXII activation in human plasma. In NHP, Aβ42
induced FXII activation and HK cleavage, and 3E8 completely blocked HK cleavage36 (Figs 1-3). Although significant, 3E8 did not completely prevent FXII activation (Fig 3D, 3E & Fig 4). Using KN-DF human plasma, we found that Aβ42 induced a small but significant amount of FXII activation (Lane 6 of Fig 4A, Fig 4B). 3E8 did not have a significant effect on Aβ42-induced FXII activation in KN-DF plasma (Lanes 6 vs 7 in Fig 4A, Fig B). The addition of 3E8 to normal plasma and the overall lack of HK in KN-DF plasma have similar inhibitory effects on Aβ42-induced FXII activation (Lanes 3 vs 6 in Fig 4A, Fig 4B). These results indicate that HK plays an important role in Aβ42-induced FXII activation and that HK participates in contact system activation primarily through the 20-residue region of D6. Therefore, an antibody blocking this region, like 3E8, is effective in inhibiting contact system activation.

**3E8 delays intrinsic coagulation and prevents Aβ42-induced intrinsic coagulation.**

FXI plays an important role in the activation of the intrinsic coagulation pathway52. Since 3E8 and FXI both bind D6 of HK, 3E8 might interfere with FXI binding and impact intrinsic coagulation. To analyze whether 3E8 affects intrinsic coagulation, we measured aPTT53. After incubating human plasma with various concentrations of 3E8, we induced intrinsic coagulation. While very low dose of 3E8 (0.05 μM) did not impact aPTT, a higher dose (0.15 μM) significantly delayed aPTT (Fig 5A). We also analyzed the effect of 3E8 on the extrinsic coagulation pathway measuring PT53. We found that 3E8 did not impact the PT (Fig 5B), indicating that 3E8 delays intrinsic, but not extrinsic, coagulation.
Aβ42 induces thrombin generation and dramatically accelerated clotting in normal plasma (Fig. 5C). To analyze whether 3E8 anti-HK antibody could prevent Aβ42-induced clotting, we incubated human plasma with various concentrations of 3E8 and then Aβ42 before adding calcium to initiate coagulation. 3E8 (75 nM) abolished the effects of Aβ42-induced accelerated clotting and normalized clotting (Fig 5C).

Since FXI can also be activated by thrombin, we used a purified protein system to examine whether 3E8 could affect thrombin-induced FXI activation. Our results showed that 3E8 anti-HK antibody did not inhibit FXI activation by thrombin (Fig S1).

**Aβ42 binding to both HK and FXII, and PK and FXI binding to HK play important roles in Aβ42-induced plasma contact system activation.**

We analyzed the interaction between Aβ42 and other contact system proteins in NHP using biotinylated-Aβ42 (B-Aβ42). Molecules were pulled down and analyzed by non-reducing SDS-PAGE/Western blots. The specificity of the antibodies used was validated using purified proteins (Lanes 1-4, Fig 6A). B-Aβ42 pulled down FXII/FXIIa, cHK, some FXI, and some PK (Lane 7, Fig 6A). Since the contact system was activated by Aβ42 during the pull-down process: FXII was dramatically activated; HK was cleaved; PK and FXI were decreased due to their activation to PKa and FXIa, respectively, not detectable by these antibodies (compare lanes 5-7, Fig 6A). Most FXIIa was pulled down by Aβ42, as FXIIa was not detected in the supernatant after B-Aβ42 pulldown. Furthermore, cHK was pulled down by Aβ42 (Lanes 5-7, Fig 6A). These results show that activated FXII and cleaved HK bind to Aβ42. Transferrin (TF), a non-contact system
plasma protein used for normalization, was not pulled down by B-Aβ42, indicating the specificity of the pulldown assay.

Since the pull-down process with B-Aβ42 induced contact system activation, we used FXII-deficient human plasma (FXII-DF) for further studies. All proteins except FXII were detected in FXII-DF (Lane 1, Fig 6B). B-Aβ42 pulled down HK, FXI, and PK, but not TF (Lane 2, Fig 6B). 3E8 blocked FXI and PK pulldown by B-Aβ42, but not HK (Lanes 2 vs 3, Fig 6B), indicating FXI and PK pull-down by B-Aβ42 depends on their binding to HK. 3E8 blocked FXI and PK binding to HK, therefore preventing FXI and PK pull-down. FXII-deficiency prevented Aβ42-induced HK cleavage as cHK was not detected (Fig 6A vs 6B), indicating that FXII is necessary for Aβ42-induced contact system activation.

To further analyze the role of HK in FXI/PK’s interaction with Aβ42, KN-DF plasma was used. All proteins analyzed except HK were detected in KN-DF plasma, and the protein levels were similar between NHP and KN-DF plasma (Lanes 1 and 2, Fig 6C). B-Aβ42 pulled down FXII but neither FXI nor PK from KN-DF plasma (Lane 3, Fig 6C). This result further shows that FXI and PK pulled down by Aβ42 depends on HK; in the absence of HK, Aβ42 did not pull down FXI and PK. Even though Aβ42 induced FXII activation and pulled down FXIIa in NHP (Lane 7, Fig 6A), it did not pull down FXIIa in KN-DF plasma (Lane 3, Fig 6C) as there was likely too little FXIIa formed to be pulled down (Fig 4). This result indicates that Aβ42 did not induce FXII activation in KN-DF plasma. Therefore, Aβ42 binding to both FXII and HK is critical for Aβ42-mediated contact system activation.

To further investigate the role of 3E8 anti-HK antibody in FXI and PK binding to HK, we used an ELISA-based binding assay. Plates were coated with PK (Fig 6D) or FXI (Fig
6E) and then incubated with HK in the presence or absence of 3E8. In the absence of 3E8, HK bound to PK (Fig 6D) or FXI (Fig 6E). In the presence of 3E8, HK binding to PK (Fig 6D) or FXI (Fig 6D) was blocked. Control IgG did not influence HK binding to PK or FXI. These results directly show that 3E8 blocked PK or FXI binding to HK.

**3E8 anti-HK antibody disassembles PK/HK and FXI/HK complexes in NHP in the absence of a contact system activator ex vivo.**

Since PK and FXI circulate in the blood as complexes with HK, we hypothesized that 3E8 could disassemble PK/HK or FXI /HK complexes in NHP in the absence of contact system activators. To test this possibility, we incubated NHP with 3E8 anti-HK antibody, then pulled down HK and its bound proteins with an anti-HK antibody that does not recognize or bind D6 (biotinylated-2B7, B-2B7)⁴⁴. In the absence of 3E8, B-2B7 anti-HK antibody pulled down HK, FXI, and PK (Lanes 1 and 3 in Fig 7A). However, in the presence of 3E8, B-2B7 antibody pulled down HK but not FXI or PK (Lane 2 in Fig 7A). This result indicates that the 3E8 anti-HK antibody disassembled PK/HK and FXI/HK complexes in human plasma in the absence of contact system activation ex vivo. There was significantly more intact HK pulled down in the presence of 3E8 compared to in the absence of 3E8 (Fig 7A & 7B). Control IgG did not have an effect on the pulldown, indicating the specificity of the 3E8 antibody. Neither FXII nor albumin were pulled down, demonstrating the specificity of the B-2B7 antibody for HK and the lack of binding between FXII and HK in the absence of a contact system activator. Overall, this experiment shows that the 3E8 anti-HK antibody is capable of disassembling PK/HK and FXI/HK complexes in normal human plasma in the absence of contact system activation ex vivo.
Discussion

HK is a nonenzymatic cofactor for the optimal activation of the plasma contact system. HK mostly circulates in the blood complexed with PK or FXI\(^{55,56}\). FXII binds directly to negatively charged surfaces, which can lead to autoactivation, whereas HK may be necessary for optimal activation of PK and FXI by surface-bound activated FXIIa and for positive feedback activation of FXII by kallikrein (PKa)\(^{57,58}\). Plasma deficient in HK has a markedly prolonged aPTT \textit{ex vivo}\(^{59-62}\).

Human HK consists of six domains (designated D1 to D6, respectively). Previous studies revealed that D6 of HK contains the PK and FXI binding sites, which partially overlap\(^{47-50}\). This region is important for HK-dependent clotting activity of normal human plasma and dextran sulfate sodium salt (DXS)-induced activation of PK and HK cleavage\(^{47}\). We focused on a smaller 20-amino acid region within the identified PK/FXI binding site in D6 of HK\(^{47-49}\). The effect of 3E8 anti-HK antibody on Aβ42-induced HK cleavage is dose-dependently blocked by the HK-D6 peptide (Fig. 2), indicating the importance of this region. Moreover, 3E8 inhibited FXII and PK activation and delayed aPTT in normal human plasma. Our immunoprecipitation experiment in human plasma showed that 3E8 directly bound HK yet bound neither FXII nor PK, indicating the effects of 3E8 on contact system activation is through its binding to HK. Together, these results suggest the effects of 3E8 anti-HK antibody on Aβ42-induced contact system activation is through its binding to a 20-amino acid region of HK’s D6, demonstrating that this small region of HK plays an important role in Aβ42-induced contact system activation.
Based on previous studies from other investigators and our present results, we hypothesize the following models: In normal plasma (Fig S2A), PK and FXI bind to HK and form complexes. Aβ42 binds to FXII and activates FXII to FXIIa, which initiates contact system activation. Aβ42 also binds to HK, which facilitates access of PK and FXII to Aβ42-bound FXIIa (Fig 6A). FXIIa activation of FXI (to FXIa) triggers the intrinsic coagulation pathway, leading to clot formation, whereas FXIIa activation of PK (to PKa/kallikrein) leads to the cleavage of HK and release of bradykinin from its precursor HK and subsequent binding to bradykinin receptors and initiation of inflammatory processes. PKa can positively feed-back to activate FXII, and FXIIa can further activate the downstream pathways and amplify contact system activation. In the presence of 3E8 (Fig S2B), 3E8 binds to D6 of HK and blocks PK and FXI binding to HK (Fig 6B, 6D, & 6E). Aβ42 can minimally activate FXII in the presence of 3E8 (Fig 4), but Aβ42-bound FXIIa does not have access to FXI and PK due to 3E8’s binding and therefore cannot activate PK or FXI. Since PK is not activated, it cannot feedback to activate more FXII, and FXII remains minimally activated by Aβ42 (Fig 3D). The effect of KN-DF is similar to that of 3E8. In the absence of HK (Fig S2C), Aβ42 can bind to FXII and initiate limited FXII activation (Fig 4), but Aβ42-bound FXIIa does not have access to FXI and PK and therefore cannot activate PK nor FXI. Since PK is not activated, it cannot positively feed-back to activate FXII, and FXII has very limited activation by Aβ42 (Fig 4 and Fig 6C). Therefore, in the absence of HK, contact system activation is very limited by Aβ42 (Fig 4).
HK has important functions in many pathophysiological conditions\textsuperscript{39,63-65}. Kininogen-1 knockout mice are protected from ischemic neurodegeneration without an increase in infarct-associated hemorrhage\textsuperscript{66}. Therefore, HK is instrumental in pathologic thrombus formation and inflammation but dispensable for hemostasis\textsuperscript{66}. Bradykinin, which is released from HK, is involved in many pathologies in a variety of systems and organs\textsuperscript{40,67-74}. These studies suggest that targeting HK may be beneficial in many pathological conditions. Our study showed that 3E8, an antibody targeting HK, not only blocks bradykinin release\textsuperscript{36} and subsequent inflammatory activity, but it also inhibits FXI and PK activation, prevents FXII feedback activation of FXII, and prevents intrinsic coagulation \textit{ex vivo}. Furthermore, 3E8 can disassemble HK/PK and HK/FXI complexes in the absence of a contact system activator. Our studies may provide important information for novel therapeutic and prophylactic strategies for contact system-related pathological conditions, such as Alzheimer’s disease and hereditary angioedema.
Acknowledgments:

The authors thank members of the Strickland laboratory for helpful discussions. This work was supported by NIH grants NS102721 and AG069987, Cure Alzheimer’s Fund, Alzheimer’s Association, Robertson Therapeutic Development Fund, Samuel Newhouse Foundation, Mr. John Herrmann, and Rudin Family Foundation. It was also supported by UL1TR001866 from the National Center for Advancing Translational Sciences, NIH Clinical and Translational Science Award program.

Authorship contributions:

Z.L.C. designed the study, performed experiments, analyzed data, and wrote the manuscript; P.S. and K.H. performed experiments and analyzed data; S.S. helped design the study and analyze data; E.H.N. designed the study, participated in data analysis, and wrote the manuscript.

Conflict-of-interest disclosure: The 3E8 and 2B7 anti-HK antibodies have been licensed to Millipore Sigma.
1. Iturria-Medina Y, Sotero RC, Toussaint PJ, Mateos-Perez JM, Evans AC. Early role of vascular dysregulation on late-onset Alzheimer's disease based on multifactorial data-driven analysis. Nat Commun. 2016;7:11934.
2. Lee S, Viqar F, Zimmerman ME, et al. White matter hyperintensities are a core feature of Alzheimer's disease: Evidence from the dominantly inherited Alzheimer network. Ann Neurol. 2016.
3. Wyss-Coray T, Rogers J. Inflammation in Alzheimer disease—a brief review of the basic science and clinical literature. Cold Spring Harb Perspect Med. 2012;2(1):a006346.
4. Nation DA, Sweeney MD, Montagne A, et al. Blood-brain barrier breakdown is an early biomarker of human cognitive dysfunction. Nat Med. 2019;25(2):270-276.
5. Farkas E, Luiten PG. Cerebral microvascular pathology in aging and Alzheimer's disease. Prog Neurobiol. 2001;64(6):575-611.
6. Kalaria RN. Small vessel disease and Alzheimer's dementia: pathological considerations. Cerebrovasc Dis. 2002;13 Suppl 2:48-52.
7. HumpeL C. Chronic mild cerebrovascular dysfunction as a cause for Alzheimer's disease? Exp Gerontol. 2011;46(4):225-232.
8. Hyman BT, Phelps CH, Beach TG, et al. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. Alzheimers Dement. 2012;8(1):1-13.
9. Cortes-Canteli M, Mattei L, Richards AT, Norris EH, Strickland S. Fibrin deposited in the Alzheimer's disease brain promotes neuronal degeneration. Neurobiol Aging. 2015;36(2):608-617.
10. Cortes-Canteli M, Zamolodchikov D, Ahn HJ, Strickland S, Norris EH. Fibrinogen and altered hemostasis in Alzheimer's disease. J Alzheimers Dis. 2012;32(3):599-608.
11. Cruz Hernandez JC, Bracko O, Kersbergen CJ, et al. Neutrophil adhesion in brain capillaries reduces cortical blood flow and impairs memory function in Alzheimer's disease mouse models. Nat Neurosci. 2019;22(3):413-420.
12. de la Torre JC. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. Lancet Neurol. 2004;3(3):184-190.
13. Kisler K, Nelson AR, Montagne A, Zlokovic BV. Cerebral blood flow regulation and neurovascular dysfunction in Alzheimer disease. Nat Rev Neurosci. 2017;18(7):419-434.
14. Strickland S. Blood will out: vascular contributions to Alzheimer's disease. J Clin Invest. 2018;128(2):556-563.
15. Suidan GL, Singh PK, Patel-Hett S, et al. Abnormal clotting of the intrinsic/contact pathway in Alzheimer disease patients is related to cognitive ability. Blood Adv. 2018;2(9):954-963.
16. Sweeney MD, Montagne A, Sagare AP, et al. Vascular dysfunction-The disregarded partner of Alzheimer's disease. Alzheimers Dement. 2019;15(1):158-167.
17. Sweeney MD, Sagare AP, Zlokovic BV. Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. Nat Rev Neurol. 2018;14(3):133-150.
18. Singh PK, Chen ZL, Ghosh D, Strickland S, Norris EH. Increased plasma bradykinin level is associated with cognitive impairment in Alzheimer's patients. Neurobiol Dis. 2020;139:104833.
19. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol. 2012;34(1):43-62.
20. Flick MJ, LaJeunesse CM, Talmage KE, et al. Fibrin(ogen) exacerbates inflammatory joint disease through a mechanism linked to the integrin alphaMbeta2 binding motif. *J Clin Invest.* 2007;117(11):3224-3235.

21. Akassoglou K, Strickland S. Nervous system pathology: the fibrin perspective. *Biol Chem.* 2002;383(1):37-45.

22. Cortes-Canteli M, Paul J, Norris EH, et al. Fibrinogen and beta-amyloid association alters thrombosis and fibrinolysis: a possible contributing factor to Alzheimer's disease. *Neuron.* 2010;66(5):695-709.

23. Merlini M, Rafalski VA, Rios Coronado PE, et al. Fibrinogen Induces Microglia-Mediated Spine Elimination and Cognitive Impairment in an Alzheimer’s Disease Model. *Neuron.* 2019;101(6):1099-1108 e1096.

24. Ryu JK, Rafalski VA, Meyer-Franke A, et al. Fibrin-targeting immunotherapy protects against neuroinflammation and neurodegeneration. *Nat Immunol.* 2018;19(11):1212-1223.

25. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. *J Thromb Haemost.* 2016;14(1):28-39.

26. Viel TA, Buck HS. Kallikrein-kinin system mediated inflammation in Alzheimer’s disease in vivo. *Curr Alzheimer Res.* 2011;8(10):1244-1260.

27. Greenwald J, Riek R. Biology of amyloid: structure, function, and regulation. *Structure.* 2010;18(10):1244-1260.

28. Musiek ES, Holtzman DM. Three dimensions of the amyloid hypothesis: time, space and 'wingmen'. *Nat Neurosci.* 2015;18(6):800-806.

29. Selkoe DJ, Hardy J. The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med.* 2016.

30. Maas C, Govers-Riemslag JW, Bouma B, et al. Misfolded proteins activate factor XII in humans, leading to kallikrein formation without initiating coagulation. *J Clin Invest.* 2008;118(9):3208-3218.

31. Shibayama Y, Joseph K, Nakazawa Y, Ghebreihiwet B, Peerschke EI, Kaplan AP. Zinc-dependent activation of the plasma kinin-forming cascade by aggregated beta amyloid protein. *Clin Immunol.* 1999;90(1):89-99.

32. Zamolodchikov D, Renne T, Strickland S. The Alzheimer’s disease peptide beta-amyloid promotes thrombin generation through activation of coagulation factor XII. *J Thromb Haemost.* 2016;14(5):995-1007.

33. Yang L, Li Y, Bhattacharya A, Zhang Y. A plasma proteolysis pathway comprising blood coagulation proteases. *Oncotarget.* 2016;7(27):40919-40938.

34. Renne T. The Factor XII-Driven Plasma Contact System. In: Marder VJ, Aird WC, Bennett JS, Schulman S, White GC, eds. Hemostasis and Thrombosis: Basic Principles and Clinical Practice. Philadelphia: Wolters Kluwer/Lippincott, Williams & Wilkins; 2013:242-253.

35. Chen ZL, Revenko AS, Singh P, MacLeod AR, Norris EH, Strickland S. Depletion of coagulation factor XII ameliorates brain pathology and cognitive impairment in Alzheimer disease mice. *Blood.* 2017;129(18):2547-2556.

36. Chen ZL, Singh P, Wong J, Horn K, Strickland S, Norris EH. An antibody against HK blocks Alzheimer's disease peptide beta-amyloid-induced bradykinin release in human plasma. *Proc Natl Acad Sci U S A.* 2019;116(46):22921-22923.

37. Maas C, Renne T. Coagulation factor XII in thrombosis and inflammation. *Blood.* 2018;131(17):1903-1909.

38. Murugesan N, Fickweiler W, Clermont AC, Zhou Q, Feener EP. Retinal proteome associated with bradykinin-induced edema. *Exp Eye Res.* 2019;186:107744.

39. Wu Y. Contact pathway of coagulation and inflammation. *Thromb J.* 2015;13:17.
40. Ashby EL, Love S, Kehoe PG. Assessment of activation of the plasma kallikrein-kinin system in frontal and temporal cortex in Alzheimer’s disease and vascular dementia. *Neurobiol Aging*. 2012;33(7):1345-1355.

41. Yasuhara O, Walker DG, McGeer PL. Hageman factor and its binding sites are present in senile plaques of Alzheimer’s disease. *Brain Res*. 1994;654(2):234-240.

42. Bergamaschini L, Parnetti L, Pareyson D, Canziani S, Cugno M, Agostoni A. Activation of the contact system in cerebrospinal fluid of patients with Alzheimer disease. *Alzheimer Dis Assoc Disord*. 1998;12(2):102-108.

43. Zamolodchikov D, Chen ZL, Conti BA, Renne T, Strickland S. Activation of the factor XII-driven contact system in Alzheimer’s disease patient and mouse model plasma. *Proc Natl Acad Sci U S A*. 2015;112(13):4068-4073.

44. Yamamoto-Imoto H, Zamolodchikov D, Chen ZL, et al. A novel detection method of cleaved plasma high-molecular-weight kininogen reveals its correlation with Alzheimer’s pathology and cognitive impairment. *Alzheimers Dement (Amst)*. 2018;10:480-489.

45. Joseph K, Tholanikunnel BG, Kaplan AP. Factor XII-independent cleavage of high-molecular-weight kininogen by prekallikrein and inhibition by C1 inhibitor. *J Allergy Clin Immunol*. 2009;124(1):143-149.

46. Chen ZL, Yao Y, Norris EH, et al. Ablation of astrocytic laminin impairs vascular smooth muscle cell function and leads to hemorrhagic stroke. *J Cell Biol*. 2013;202(2):381-395.

47. Reddigari SR, Kaplan AP. Monoclonal antibody to human high-molecular-weight kininogen recognizes its prekallikrein binding site and inhibits its coagulant activity. *Blood*. 1989;74(2):695-702.

48. Tait JF, Fujikawa K. Identification of the binding site for plasma prekallikrein in human high molecular weight kininogen. A region from residues 185 to 224 of the kininogen light chain retains full binding activity. *J Biol Chem*. 1986;261(33):15396-15401.

49. Tait JF, Fujikawa K. Primary structure requirements for the binding of human high molecular weight kininogen to plasma prekallikrein and factor XI. *J Biol Chem*. 1987;262(24):11651-11656.

50. Thompson RE, Mandle R, Jr., Kaplan AP. Studies of binding of prekallikrein and Factor XI to high molecular weight kininogen and its light chain. *Proc Natl Acad Sci U S A*. 1979;76(10):4862-4866.

51. Renne T, Stavrou EX. Roles of Factor XII in Innate Immunity. *Front Immunol*. 2019;10:2011.

52. Emsley J, McEwan PA, Gailani D. Structure and function of factor XI. *Blood*. 2010;115(13):2569-2577.

53. Winter WE, Flax SD, Harris NS. Coagulation Testing in the Core Laboratory. *Lab Med*. 2017;48(4):295-313.

54. Matafonov A, Sarilla S, Sun MF, et al. Activation of factor XI by products of prothrombin activation. *Blood*. 2011;118(2):437-445.

55. Mandle RJ, Colman RW, Kaplan AP. Identification of prekallikrein and high-molecular-weight kininogen as a complex in human plasma. *Proc Natl Acad Sci U S A*. 1976;73(11):4179-4183.

56. Thompson RE, Mandle R, Jr., Kaplan AP. Association of factor XI and high molecular weight kininogen in human plasma. *J Clin Invest*. 1977;60(6):1376-1380.

57. Griffin JH, Cochrane CG. Mechanisms for the involvement of high molecular weight kininogen in surface-dependent reactions of Hageman factor. *Proc Natl Acad Sci U S A*. 1976;73(8):2554-2558.

58. Meier HL, Pierce JV, Colman RW, Kaplan AP. Activation and function of human Hageman factor. The role of high molecular weight kininogen and prekallikrein. *J Clin Invest*. 1977;60(1):18-31.

59. Colman RW, Bagdasarian A, Talamo RC, et al. Williams trait. Human kininogen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with abnormalities of the Hageman factor-dependent pathways. *J Clin Invest*. 1975;56(6):1650-1662.

60. Donaldson VH, Glueck HI, Miller MA, Movat HZ, Habal F. Kininogen deficiency in Fitzgerald trait: role of high molecular weight kininogen in clotting and fibrinolysis. *J Lab Clin Med*. 1976;87(2):327-337.
61. Saito H, Ratnoff OD, Waldmann R, Abraham JP. Fitzgerald Trait: Deficiency of a Hitherto Unrecognized Agent, Fitzgerald Factor, Participating in Surface-Mediated Reactions of Clotting, Fibrinolysis, Generation of Kinins, and the Property of Diluted Plasma Enhancing Vascular Permeability (PF/Dil). J Clin Invest. 1975;55(5):1082-1089.
62. Wuepper KD, Miller DR, Lacombe MJ. Flaujeac trait. Deficiency of human plasma kininogen. J Clin Invest. 1975;56(6):1663-1672.
63. Wu Y, Dai J, Schmuckler NG, et al. Cleaved high molecular weight kininogen inhibits tube formation of endothelial progenitor cells via suppression of matrix metalloproteinase 2. J Thromb Haemost. 2010;8(1):185-193.
64. Yang A, Dai J, Xie Z, et al. High molecular weight kininogen binds phosphatidylserine and opsonizes urokinase plasminogen activator receptor-mediated efferocytosis. J Immunol. 2014;192(9):4398-4408.
65. Yang A, Xie Z, Wang B, Colman RW, Dai J, Wu Y. An essential role of high-molecular-weight kininogen in endotoxemia. J Exp Med. 2012;214(9):2649-2670.
66. Langhauser F, Gob E, Kraft P, et al. Kininogen deficiency protects from ischemic neurodegeneration in mice by reducing thrombosis, blood-brain barrier damage, and inflammation. Blood. 2012;120(19):4082-4092.
67. Albert-Weissenberger C, Siren AL, Kleinschnitz C. Ischemic stroke and traumatic brain injury: the role of the kallikrein-kinin system. Prog Neurobiol. 2013;101-102:65-82.
68. Gauberti M, Potzeha F, Vivien D, Martinez de Lizarrondo S. Impact of Bradykinin Generation During Thrombolysis in Ischemic Stroke. Front Med (Lausanne). 2018;5:195.
69. Isordia-Salas I, Pixley RA, Sainz IM, Martinez-Murillo C, Colman RW. The role of plasma high molecular weight kininogen in experimental intestinal and systemic inflammation. Arch Med Res. 2005;36(1):87-95.
70. Liu J, Feener EP. Plasma kallikrein-kinin system and diabetic retinopathy. Biol Chem. 2013;394(3):319-328.
71. Nicola H. The role of contact system in septic shock: the next target? An overview of the current evidence. J Intensive Care. 2017;5:31.
72. Nokkari A, Abou-El-Hassan H, Mechref Y, et al. Implication of the Kallikrein-Kinin system in neurological disorders: Quest for potential biomarkers and mechanisms. Prog Neurobiol. 2018;165-167:26-50.
73. Sala-Cunill A, Bjorkqvist J, Senter R, et al. Plasma contact system activation drives anaphylaxis in severe mast cell-mediated allergic reactions. J Allergy Clin Immunol. 2015;135(4):1031-1043 e1036.
74. Simao F, Ustunkaya T, Clermont AC, Feener EP. Plasma kallikrein mediates brain hemorrhage and edema caused by tissue plasminogen activator therapy in mice after stroke. Blood. 2017;129(16):2280-2290.
Figure legends

Figure 1. Characterization of the 3E8 anti-HK antibody.

(A) Western blot shows 3E8 specifically recognizes HK in normal human plasma and cHK in Aβ42-treated human plasma. Purified human HK and cHK were included as controls. In kininogen-deficient (KN-DF) human plasma, HK was not detected by 3E8, indicating the specificity of 3E8. The membrane was stripped and reprobed with anti-LMWK antibody. LMWK was detected in normal and Aβ42-treated human plasma but not in KN-DF plasma. 3E8 did not recognize LMWK. (B) The 3E8 and 2B7 anti-HK antibodies pulled down HK, but not FXII, PK, or transferrin (TF) from human plasma. Human plasma was incubated with biotinylated 3E8, 2B7, and 4B12 anti-HK antibodies and control IgG, and streptavidin was added to pull down the antibody-antigen complex. The samples were analyzed by Western blot using commercial antibodies against HK, FXII, PK, and TF. The 3E8 and 2B7 HK antibodies immunoprecipitated HK from human plasma, but they did not pull down FXII, PK, or TF. The 4B12 antibody, which recognizes cleaved HK but not intact HK, did not pull down HK, FXII, PK, or TF from human plasma. (C) HK ELISA shows the K_D of 3E8 anti-HK antibody binding to HK is 168±38 pM. The experiments were performed in triplicate and repeated 3 times.

Figure 2. The inhibitory effects of the 3E8 anti-HK antibody on Aβ42-induced HK cleavage is abolished by competitive HK-D6 peptide.

(A) EDTA-human plasma was incubated with buffer, 3E8 anti-HK antibody (0.5 μM), control IgG (0.5 μM), HK-D6 (0.5 μM, 1 μM, 2 μM), 3E8 anti-HK antibody (0.5μM) + HK-
D6 (0.5 μM, 1 μM, 2 μM) or control IgG (0.5 μM) + HK-D6 (0.5 μM, 1 μM, 2 μM) (as indicated in A) at 37°C for 20 minutes. Aβ42 (5 μM) was added and incubated for two hours. Western blotting analyses were performed with antibodies against HK and TF. 3E8 HK antibody protected HK from Aβ42-induced cleavage (Lane 3), but control IgG (Lane 4) and different concentrations of HK-D6 did not have significant effects on Aβ42-induced HK cleavage (Lanes 5-7, and B). However, HK-D6 dose-dependently inhibited the protective effects of 3E8 anti-HK antibody on Aβ42-induced HK cleavage (Lanes 1, 3, 8-10, and B). HK-D6 did not have significant effects on the control IgG in Aβ42-induced HK cleavage (Lanes 4, 11-13, and B). HK Western blotting signals were normalized to TF. n=6, Data are denoted as mean ± SEM. *P <0.05, **P ≤ 0.01, ***P ≤ 0.001. P > 0.05 was not significant (n.s.).

Figure 3. 3E8 dose-dependently inhibits Aβ42-induced PK and FXII activation in human plasma.

(A & B) The 3E8 anti-HK antibody dose-dependently inhibited Aβ42-induced PK activation in normal human plasma (NHP). NHP was incubated with buffer, 3E8 anti-HK antibody (0.02 μM, 0.1 μM, 0.5 μM), or control IgG (0.5 μM) at 37°C for 20 minutes. Aβ42 (5 μM) was added and incubated at 37°C for two hours (A). Western blots were performed with antibodies against HK, PK, FXII, and TF. 3E8 dose-dependently protected HK from Aβ42-induced cleavage as shown previously36. It also dose-dependently inhibited PK activation. Decreases in PK band indicates activation of PK to PKa; PKa was not detectable by this antibody. (Lanes 1-5 in A, and B) Control IgG did not show any effect on Aβ42-induced PK activation (Lane 6 in A, and B). (C) 3E8 dose-
dependently inhibited Aβ42-induced kallikrein activity in human plasma. NHP was incubated with buffer, control IgG (50 nM), or various concentrations of 3E8 HK antibody (5 nM, 25 nM, and 50 nM) at 37°C for 20 minutes. Aβ42 (5 μM) and chromogenic substrate S-2302 (0.67 mM final concentration) were added, and absorbance at 405 nm was read for 60 min at 37°C. In the absence of 3E8, Aβ42-induced a dramatic increase in kallikrein activity. The 3E8 antibody dose-dependently inhibited Aβ42-induced kallikrein activation, while control IgG had no effect. (D & E) 3E8 anti-HK antibody dose-dependently inhibited FXII activation. 3E8 also dose-dependently inhibited FXII activation (FXIIa) (Lanes 1-5 in D, and E), while control IgG did not influence Aβ42-induced FXII activation (Lane 6 in D, and E). FXIIa and PK Western blots were normalized against TF. n=6, Data are denoted as mean ± SEM. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001. P > 0.05 was not significant (n.s.).

Figure 4. 3E8 anti-HK antibody has the same effects on Aβ42-induced FXII activation as does the absence of HK in human plasma.

Normal human plasma (NHP) or KN-DF human plasma were incubated with buffer, 3E8 (0.5 μM), or control IgG (0.5 μM) at 37°C for 20 minutes. Then Aβ42 (5 μM) was added and incubated at 37°C for two hours. Western blotting analyses were performed with antibodies against FXII/FXIIa, HK, and transferrin (TF). In the absence of 3E8, Aβ42 induced FXII activation and HK cleavage in normal plasma (Lane 2 in A, and B). It also induced a small but significant activation of FXII in KN-DF human plasma (Lane 6 in A, and B). 3E8 blocked FXII activation in normal plasma but had no significant effect on FXII activation in KN-DF plasma (Lane 7 in A, and B). Control IgG did not affect Aβ42-
induced FXII or HK changes. FXIIa and HK signals were normalized to transferrin (TF). n=6, Data are denoted as mean ± SEM. *P < 0.05, ***P ≤ 0.001. P > 0.05 was not significant (n.s.).

Figure 5. The 3E8 anti-HK antibody delays aPTT but not PT and prevents Aβ42-induced intrinsic coagulation in human plasma.

(A) The 3E8 HK antibody delayed aPTT. Citrated-human plasma was incubated with buffer, 3E8 (0.05 μM, 0.15 μM, 0.75 μM, 3.75 μM), or control IgG (3.75 μM) at 37ºC for 20 minutes. Intrinsic coagulation was initiated by adding aPTT reagent and CaCl₂ solution. Absorbance readings were recorded every 5 seconds at 350 nm, and aPTT was determined. 3E8 did not significantly affect aPTT at 0.05 μM, but it significantly delayed the aPTT at 0.15 μM. However, higher amounts of 3E8 did not cause further delay. Control IgG did not affect aPTT. (B) The 3E8 HK antibody did not affect PT. Citrated-human plasma was incubated with buffer, 3E8 (3.75 μM), or control IgG (3.75 μM) at 37ºC for 20 minutes. The extrinsic coagulation pathway was initiated by adding tissue factor with CaCl₂. Absorbance readings were recorded every 5 seconds at 350 nm, and PT was determined. (C) 3E8 normalized Aβ42-induced intrinsic coagulation in human plasma. Citrated-human plasma was incubated with buffer, 3E8 (0.025 μM, 0.075 μM, 0.25 μM), or control IgG (0.25 μM) at 37ºC for 20 minutes. Aβ42 (4 μM) with CaCl₂ was added to induced coagulation. 3E8 did not significantly affect Aβ42-induced coagulation at 0.025μM, while 3E8 corrected Aβ42-induced coagulation at 0.075μM and 0.25μM. However, there was no significant difference between 0.075 μM and 0.25 μM.
3E8 antibody. Control IgG did not affect Aβ42-induced coagulation. n=6, Data are denoted as mean ± SEM. **P ≤ 0.01, ***P ≤ 0.001. P > 0.05 was not significant (n.s.).

Figure 6. Aβ42 binding to both HK and FXII, and PK and FXI binding to Hare involved in Aβ42-induced plasma contact system activation.

(A) Aβ42 pulls down FXII, cHK, FXI, and PK from pooled normal plasma (NHP). NHP was incubated with biotinylated Aβ42 (B-Aβ42). The B-Aβ42/bound protein complexes were pulled down by Dynabeads™ M-280 Streptavidin, eluted with SDS sample buffer, and analyzed by Western blot. Purified human FXII, FXI, PK, and HK were used to validate the specificity of the antibodies for each protein (Lanes 1-4). Each protein was detected in the NHP input (Lane 5) and the supernatant after B-Aβ42 pulldown (Lane 6). B-Aβ42 pulled down FXII and FXIIa (activated by Aβ42-induced contact system activation during the pull-down process), cHK (HK was cleaved due to contact system activation, so most, if not all, cHK was pulled down by Aβ42), FXI, and some PK (Lane 7). During the pulldown process, B-Aβ42 activated the contact system leading to FXII activation, decreased PK (since it was activated to PKa, which was not detectable by this antibody), FXI molecular size shift, and HK cleavage (compare Lanes 5-7). TF was not pulled down by B-Aβ42. (B) 3E8 anti-HK antibody blocked PK and FXI, but not HK, pulldown by B-Aβ42. Since the contact system was activated during the pulldown process, we used FXII-deficient (FXII-DF) human plasma to avoid B-Aβ42-induced contact system activation. All proteins except for FXII were detected in FXII-DF plasma (Lane 1). B-Aβ42 pulled down HK, FXI, and PK, but not TF (Lane 2). 3E8 blocked FXI and PK pulldown by B-Aβ42, but not HK (Lane 3). (C) HK-deficiency in KN-DF plasma
prevented FXI and PK pulldown by B-Aβ42. All proteins except HK were detected in KN-DF plasma, and protein levels were similar between PNP and KN-DF plasma (Lanes 1 vs 2). B-Aβ42 pulled down FXII but neither FXI nor PK from KN-DF plasma (Lane 3). (D, E) Plates were coated with PK (D) or FXI (E), and then incubated with HK in the presence or absence of 3E8 anti-HK antibody. In the absence of 3E8, HK binds to PK (D) or FXI (E). In the presence of 3E8, binding is blocked between HK and PK (D) or FXI (E). The control IgG did not have an effect on HK binding to PK or FXI. The experiments were performed in triplicate and repeated 3 times. Data are denoted as mean ± SEM. ***P ≤ 0.001. P > 0.05 was not significant (n.s.).

Figure 7. 3E8 anti-HK antibody disassembles PK/HK and FXI/HK complexes in normal human plasma in the absence of contact system activator ex vivo.

NHP was incubated with PBS, 3E8, or hamster IgG at 37°C for 20 min. B-2B7 anti-HK antibody was added and incubated for additional 20 min. Streptavidin was used to pull down B-2B7-bound proteins. (A) Representative Western blot results of the pulldown experiments. While HK was pulled down in all samples, FXI and PK were only pulled down in PBS- or IgG-treated plasma (Lanes 1 & 3 vs Lane 2). (B) 3E8 anti-HK antibody pulled down significantly more intact HK than PBS/IgG samples. (C & D) FXI and PK were pulled down from PBS- and hamster IgG-treated, but not 3E8-treated NHP, indicating that 3E8 anti-HK antibody is able to disassemble PK/HK and FXI/HK complexes in normal human plasma in the absence of a contact system activator ex vivo. FXII and human albumin were not pulled down in any of the samples. n=8/group. Data are denoted as mean ± SEM. **p ≤ 0.01, ****p ≤ 0.0001.
Figure 1. Characterization of 3E8 anti-HK antibody
Figure 2. The inhibitory effects of 3E8 anti-HK antibody on Aβ42-induced HK cleavage is abolished by the competitive HK-D6 peptide
Figure 3. The 3E8 anti-HK antibody dose-dependently inhibits PK and FXII activation
Figure 4. 3E8 anti-HK antibody has the same effects on Aβ42-induced FXII activation as does the absence of HK in human plasma
Figure 5. The 3E8 anti-HK antibody delays aPTT but not PT and prevents Aβ42-induced intrinsic coagulation.
Figure 6. Aβ42 binding to both HK and FXII, and PK and FXI binding to HK are involved in Aβ42-induced contact system activation.
Figure 7. 3E8 anti-HK antibody disassembles PK/HK and FXI/HK complexes in normal human plasma in the absence of a contact system activator *ex vivo*.