Proteolytic Antibody Light Chains Alter β-Amyloid Aggregation and Prevent Cytotoxicity†

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ABSTRACT: β-Amyloid (Aβ), a peptide generated by proteolytic cleavage of the amyloid precursor protein (APP), is a major constituent of the neuritic plaques associated with Alzheimer's disease (AD). Up-regulation of α-secretase, which can hydrolyze Aβ between Lys^{16} and Leu^{17}, has been proposed as a potential therapeutic strategy in the treatment of AD. Previously, we identified two light-chain antibody fragments that had proteolytic activity against Aβ, one with α-secretase-like activity and one with carboxypeptidase-like activity. Here we show that cleavage of Aβ40 by hk14, the light-chain antibody having carboxypeptidase-like activity, alters aggregation of Aβ and neutralizes any cytotoxic effects of the peptide. Cleavage of Aβ40 with c23.5, the light chain having α-secretase-like cleavage, substantially increases the aggregation rate of Aβ; however, it does not show any corresponding increase in cytotoxicity. The increase in aggregation resulting from hydrolysis by c23.5 can be attributed to the decreased solubility of the hydrolyzed products relative to the parent Aβ40, primarily the Aβ17−40 fragment. These results demonstrate that antibody fragment mediated proteolytic degradation of Aβ peptide can be a potential therapeutic route to control Aβ aggregation and toxicity in vivo. Our results also suggest that increasing α-secretase activity as a therapeutic route must be approached with some caution because this can lead to a substantial increase in aggregation.

The critical pathological features of Alzheimer's disease (AD)[1] are extracellular amyloid plaques and intracellular neurofibrillary tangles (*1*). The primary proteinaceous component of the amyloid plaques is β-amyloid (Aβ) (*2*). Aβ is generated from the amyloid precursor protein (APP) by proteolytical cleavage at both the β- and γ-secretase sites, resulting in the 40/42 amino acid products (*1*). Aβ will readily aggregate under appropriate conditions (*3*), and the different aggregate morphologies that can form, including soluble oligomers, filaments, and insoluble fibrils, have all been reported to play varying roles in the neurotoxic properties of the peptides (*1*, *4*−*7*, *8*−*13*). Aβ is a normal product of cell metabolism and is found in the plasma and cerebrospinal fluid (CSF) of healthy humans (*14*). In patients with AD however, numerous different mechanisms have been proposed to explain why Aβ forms toxic aggregates including increased production or decreased clearance of Aβ (*15*), increased oxidative stress (*16*, *17*), free-radical formation (*18*), disrupted calcium homeostasis (*19*), cytoskeletal anomalies (*20*), and metal ions (*21*). Therapeutic strategies aimed at reducing Aβ aggregation include decreasing Aβ production rates by inhibiting activity of either β- or γ-secretases, enzymes that produce the Aβ peptide (*14*), inhibiting aggregation of Aβ with β-sheet blockers or competitive inhibitors (*22*, *23*), increasing clearance of Aβ from the cerebral cortex (*24*−*27*) or from blood (*28*, *29*), and addition of chelators to bind metal ions such as Cu^{2+} and Zn^{2+} that promote aggregation (*21*, *30*). Additional therapeutic approaches include using antiinflammatory agents to decrease the inflammatory response in the brain (*31*, *32*) and using statins to reduce cholesterol levels (*33*, *34*).

Three different proteases are involved in the processing of Aβ from APP: β-secretase cleavage forms the amino terminal portion of Aβ, γ-secretase cleavage forms the carboxyl terminal portion, and α-secretase cleaves between what would be residues Lys16 and Leu17 of Aβ, precluding formation of full-length Aβ. Considerable effort has been placed to develop therapeutics that inhibit processing of APP by β- and γ-secretases or to enhance processing by α-secretase (*35*). Inhibiting either β- or γ-secretase activity has been shown to effectively decrease Aβ levels in vivo (*36*−*38*). Cleavage of APP by α-secretase prevents formation of Aβ, releasing instead a large ectodomain of APP (sAPPCα) from the cell surface that is reported to have neuroprotective and memory-enhancing properties (*39*−*41*). There is apparently a delicate balance in the brain between nonamyloidogenic cleavage of APP by α-secretase and amyloidgenic cleavage of APP by β-secretase, which when viewed in terms of the progression of AD and neurodegeneration represents a classic battle between good and bad processing of APP (*35*). Facilitating α-secretase processing of APP by supplementing

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[1] Abbreviations: AD, Alzheimer's disease; Aβ, β-amyloid; scFv, single-chain variable fragment; ThT, Thioflavin T; AFM, atomic force microscopy; MTT, 3-2,5-diphenyltetrazolium bromide.

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or increasing α-secretase activity therefore represents a potentially promising route to mitigate amyloid formation (*42*, *43*).

Degradation of Aβ by various proteolytic enzymes has been studied as a potential therapeutic route for treating AD. Neprilysin (endopeptidase 24.11) was shown to degrade both monomeric and oligomeric forms of Aβ40 (*44*−*46*), while insulin-degrading enzyme (IDE) (*47*−*49*) and endothelin-converting enzyme (ECE) have also been reported to degrade Aβ (*50*, *51*). We have previously shown that two different antibody light chains can hydrolyze Aβ: c23.5, which has α-secretase-like activity, and hk14, which has carboxypeptidase-like activity cleaving sequentially from the carboxyl terminus of Aβ (*52*). Here we extend these results to demonstrate that these proteolytic light-chain antibody fragments can alter Aβ aggregation and cytotoxicity. Proteolytic cleavage of Aβ by the hk14 antibody fragment inhibits Aβ aggregation and eliminates toxicity toward SH-SY5Y cells, while cleavage by c23.5 increases the rate and extent of Aβ aggregation but without any corresponding increase in toxicity.

EXPERIMENTAL PROCEDURES

Antibody Samples. Lyophilized samples of the c23.5 and hk14 light-chain antibodies purified from bacterial periplasmic extracts were generously provided by Dr. Sudhir Paul (University of Texas-Health Science Center, Houston, Texas). These light chains were previously reported to cleave vasoactive intestinal polypeptide (*53*). Each of the purified protein preparations contained a major ∼29 kD band corresponding to intact light chain and minor degradation products stainable with anti-human light-chain antibody (Bence Jones proteins) (*53*). The antibody fragments were lyophilized after purification and dialysis with water, stored at 4 °C, and reconstituted in 1× PBS (0.137 M NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) to a concentration of 20 μM. The purity of the c23.5 and hk14 samples were previously analyzed by SDS−PAGE, and catalytic activity toward Aβ40 was verified by mass spectrometry (MS) (*52*).

Aβ Fragments and Preparation of Aggregates. Aβ1−16 was purchased from Bachem (Torrance, CA), and Aβ40 and Aβ17−40 were purchased from Biosource (Camarillo, CA). For aggregation experiments, Aβ40 was dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanal (HFIP) to a concentration of 2 mg/mL, sonicated in a water bath for 10 min, aliquoted in microcentrifuge tubes, dried under vacuum, and stored at −20 °C. Immediately prior to use, the HFIP-treated Aβ40 was dissolved in dimethyl sulfoxide (DMSO) to 20 mg/mL and diluted to 8 μM in PBS, pH 7.4, and incubated at 37 °C without shaking in a 0.5 mL Eppendorf PCR tube. For initial studies with antibody fragment mixtures, Aβ40 was mixed to a final concentration of 8 μM, and aliquots of either c23.5 or hk14 were added at 50:1, 200:1, and 1000:1 molar dilutions. For studies using a mixture of peptide samples, Aβ17−40 with Aβ40, Aβ peptides were added in equimolar ratios to final total protein concentration of 8 μM. For the three-peptide mixture, Aβ1−16, Aβ17−40, and Aβ40, 4 μM solutions of each peptide were mixed together.

Thioflavin T (ThT) Fluorescence Assay. Fluorescence emission of ThT is shifted when it binds to β-sheet aggregate structures such as amyloid fibrils (*54*). The extent of Aβ aggregation in the samples prepared as described above was followed by periodically removing 30 μL aliquots from the samples and adding them to 2 mL of 5 μM ThT solution (50 mM phosphate buffer, pH 6.5). Fluorescence intensity was monitored at an excitation wavelength of 450 nm and an emission wavelength of 482 nm using a Shimadzu PF-3501PC spectrofluorophotometer (Shimadzu, Japan) using 1 cm light-path quartz cuvettes with both excitation and emission bandwidths of 5 nm. Each reading represented the average of three values determined by a time scan after subtracting out the fluorescence contribution from free ThT. The ThT readings obtained from samples of Aβ incubated with either hk14 or c23.5 represent the reading obtained on the sample minus the reading obtained on a control sample containing only the scFv. All ThT fluorescence experiments were performed in triplicate. The standard errors and *P* values were analyzed with Excel and MINITAB software, respectively.

Atomic Force Microscope (AFM) Imaging. APS-modified mica was used as an AFM substrate (*55*, *56*). Five microliters of sample was placed on APS-mica for 2 min, rinsed with deionized water, and dried with argon as described earlier (*55*, *56*). Images were acquired in air using a MultiMode SPM NanoScope III system (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode using silicon probes (Olympus). An analysis of the raw AFM data of the acquired images was performed using Femtoscan software. This was used as a reflection of the relative size distributions of aggregate populations in the acquired images.

MTT Assay. Human neuroblastoma cells (SH-SY5Y) were maintained in medium containing 40% minimal essential medium (MEM), 40% Ham's modification of F-12, 18% fetal bovine serum (FBS), 1% L-glutamine (3.6 mM), and 1% penicillin/streptomycin antibiotics and grown in a 5% CO_2 atmosphere at 37 °C. Cells were harvested from flasks and plated in 96-well polystyrene plates (Corning Inc., Corning, NY) with approximately 10 000 cells per 100 μL of medium per well. Plates were incubated at 37 °C for 24 h to allow cells to attach. Eight micromolar Aβ40 with or without aliquots of antibody light chains c23.5 or hk14 were preincubated for 6, 12, and 18 days before addition to cultures. Samples were diluted with fresh medium. The final concentration of Aβ40 in each culture well with or without added c23.5 or hk14 was 400 nM. Similarly, the final protein concentration in each culture well in the various peptide mixture and control samples was also 400 nM, except for the tripeptide mixture where it was 600 nM. The same volume of medium was added to all sample and control cultures. Plates were then incubated for an additional 72 h at 37 °C. Cell viability was determined using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) toxicity assay with addition of 10 μL of 5 mg/mL MTT to each well (*58*). After incubation for 3 h at 37 °C, plates were centrifuged, and medium was aspirated from each well. One hundred microliters of MTT dissolvent (0.1 N HCl in 2-propanol) was added to each well. Plates were agitated at room temperature for 15 min to dissolve crystals. The absorbance at 560 nm was measured by a Victor Wallac multiwell assay plate reader (PerkinElmer, Gaithersburg, MD). Averages from six replicate wells were used for each sample and control, and each experiment was repeated three times. Cell viability was calculated by dividing the absor-

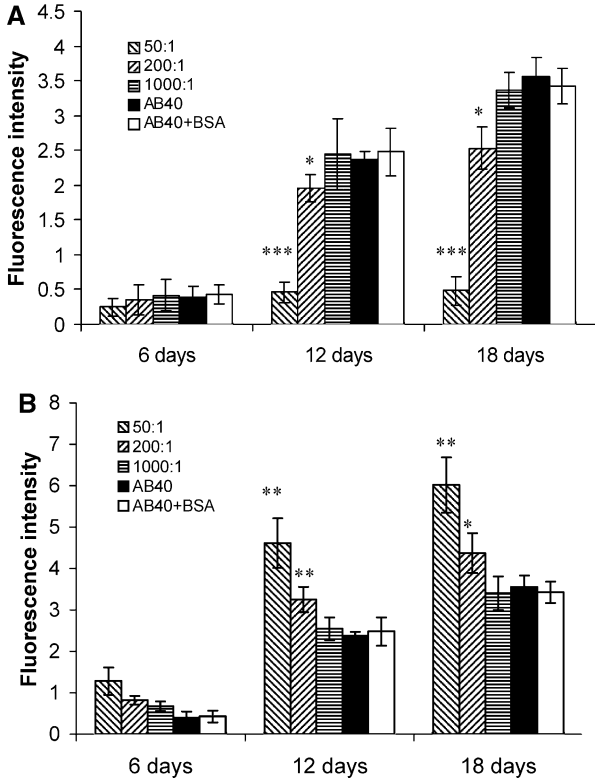

FIGURE 1: Effects on aggregation of Aβ1−40 by hk14 or c23.5. The kinetics of Aβ1−40 fibril formation was monitored by ThT fluorescence in the absence and presence of different concentrations of hk14 (A) or c23.5 (B). The samples were incubated at 37 °C, and 30 μL of sample was removed periodically and added to 2 mL of 5 μM ThT at 6, 12, and 18 days. Fluorescence intensity was measured at excitation wavelength 450 nm and emission wavelength 482 nm. The experiments were performed in triplicate. The standard errors and *P* values were analyzed with Excel and Minitab software, respectively, by one-way ANOVA (∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ compared to Aβ40 alone).

bance of wells containing samples (corrected for background) by the absorbance of wells containing medium alone (corrected for background).

RESULTS

Aβ1−40 Aggregation. Aβ1−40 will aggregate into fibrillar structures in a time-dependent manner depending on concentration and pH (*59*, *60*). Aβ1−40 when incubated alone shows the expected increase in ThT fluorescence from 0 to 6, 12, and 18 days; however when the peptide is incubated with either of the two proteolytic light-chain antibody fragments, c23.5 or hk14, the aggregation process was dramatically altered (Figure 1). Co-incubation of Aβ40 with hk14 inhibits aggregation of the peptide in a dose-dependent manner (Figure 1A). At the lowest hk14 dilution (50:1) aggregation of Aβ40 was almost totally inhibited, even after 18 days. At the higher dilution of 200:1, aggregation of Aβ40 was decreased by around 25% at each time point, while at the highest dilution tested (1000:1), no inhibition of aggregation was observed.

Essentially opposite results were obtained when Aβ40 was co-incubated with the c23.5 light-chain fragment. Here aggregation of Aβ40 increased in a dose-dependent manner, where at the lowest dilution of 50:1, there was a nearly 200% increase in aggregation at 6, 12, and 18 days, a substantially smaller increase was observed at a dilution of 200:1, and no effect was observed at the highest dilution rate (Figure 1B).

Imaging of Aggregate Morphologies. Co-incubation of Aβ40 with a 50:1 dilution of hk14 or c23.5 alters aggregation as evidenced by ThT staining and AFM imaging. AFM images of the control Aβ40 sample taken after 18 days show formation of small oligomers and extensive formation of thin filaments (Figure 2A). Images of the sample co-incubated with hk14 indicate a change in the aggregation morphology with the formation of numerous very thin, almost fragmented filaments along with the small oligomers (Figure 2B). AFM images of samples of Aβ40 co-incubated with c23.5 show extensive formation of filaments and small aggregates similar to the control sample (Figure 2C). These results are consistent with our previous study showing that Aβ1−40 when co-incubated with Aβ17−40 formed more but structurally similar filaments compared to Aβ1−40 alone (*57*). Quantification of the height distributions of the aggregates in the AFM images indicates very similar distributions of particle heights in the control Aβ alone sample and the sample co-incubated with c23.5. However when Aβ is co-incubated with hk14, there is a significant increase in the percentage of larger aggregate sizes (Table 1).

Cytotoxicity of Aβ40 Aggregate Samples. An MTT assay was used to measure the toxicity of the preincubated Aβ40 samples with and without added c23.5 or hk14 light chains after preincubation at 37 °C for 6, 12, and 18 days. When incubated alone, Aβ40 exhibited increasing cytotoxicity to SH-SY5Y cells as the preincubation time increases. Addition of a 50/1 dilution of the control protein, BSA, did not alter Aβ40 cytotoxicity. However addition of hk14 produces a dose-dependent decrease in toxicity, showing nearly complete protection against Aβ40 toxicity at a dilution of 50:1, partial protection at 200:1 dilution, and no protection at 1000:1 dilution (Figure 3A). However when Aβ40 was co-incubated with c23.5, there was a slight increase in toxicity compared to Aβ40 alone at 6 days for the higher c23.5 concentrations, but essentially no change in toxicity at 12 and 18 days at any concentration compared to Aβ40 alone (Figure 3B). Even though c23.5 results in a substantial increase in Aβ40 aggregation, it does not result in a corresponding increase in Aβ40 cytotoxicity.

Identification of c23.5 Degradation Products That Promote Aggregation. We have previously shown that the light-chain antibody fragment c23.5 can cleave Aβ40 at the α-secretase site between residues 16 and 17 and to a lesser extent can also cleave after the lysine 28 residue (*52*). Since incubation of Aβ40 with c23.5 resulted in an increase in aggregation, we tested how Aβ1−16 and Aβ17−40, the two major hydrolytic fragments resulting from α-secretase cleavage of Aβ40, would affect aggregation and cytoxicity of the parent peptide. We incubated 8 μM samples of Aβ1−16, Aβ17−40, and Aβ1−40 alone for 18 days along with mixtures containing 4 μM each of: (a) Aβ17−40 and Aβ1−40 and (b) Aβ1−16, Aβ17−40, and Aβ1−40. In addition, we incubated a control sample containing 8 μM Aβ1−40 and 0.16 μM BSA and a test sample containing 8 μM of Aβ1−40 and 0.16 μM of c23.5. Aliquots of each of these samples were periodically removed and tested for fibrillar content as determined by ThT (Figure 4A) and cytotoxicity as determined by MTT assay (Figure 4B). Aβ1−16 alone did not aggregate, nor was it cytotoxic, while

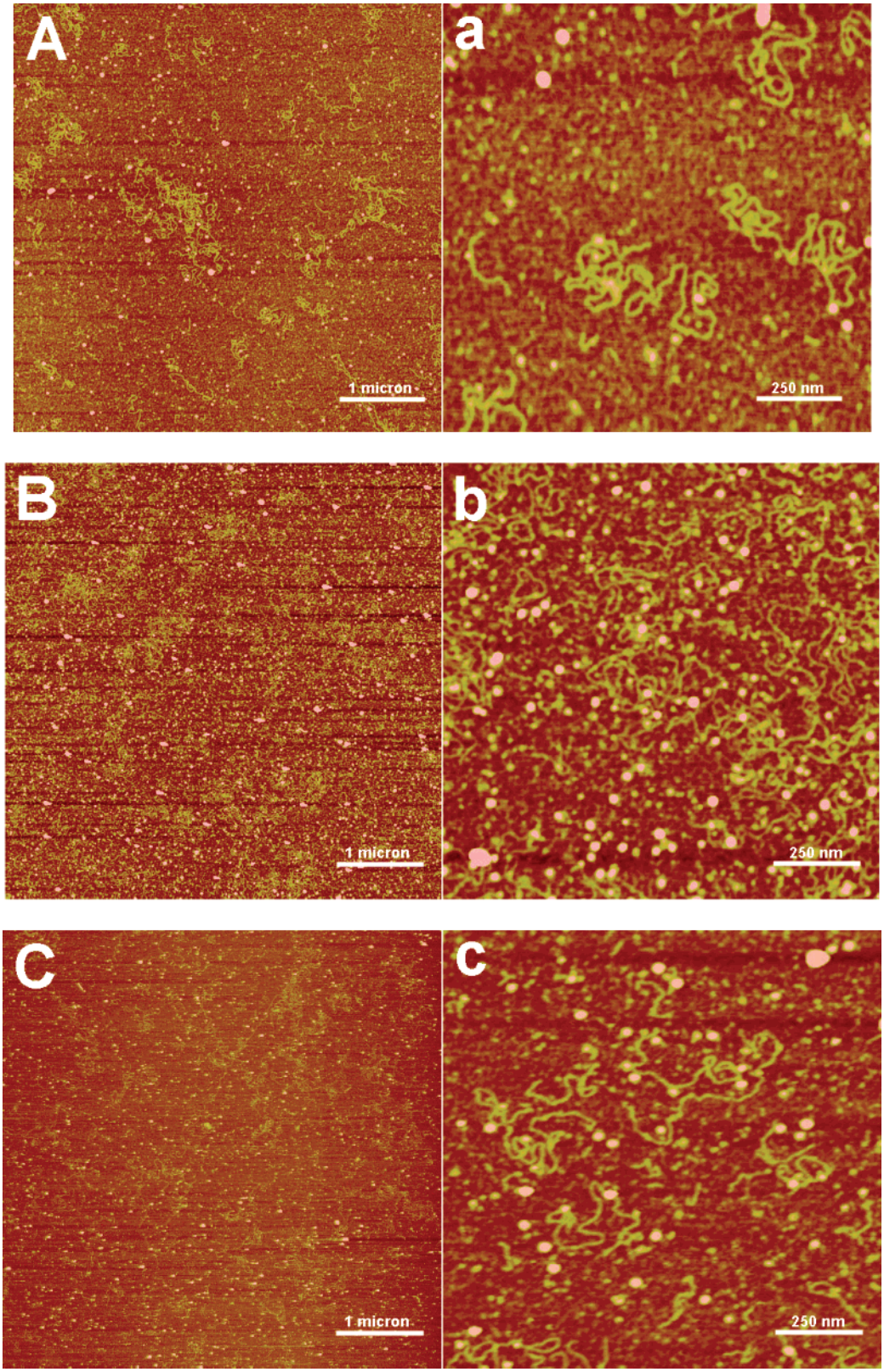

FIGURE 2: AFM imaging of Aβ40 aggregation alone and with hk14 or c23.5. AFM analysis of 18-day samples of Aβ40 aggregates when incubated alone (A), with hk14 (B), and with c23.5 (C). Scale bars = 1 μm. *Z*-scale = 250 nm. Images were acquired in air using a MultiMode SPM NanoScope III system operating in tapping mode using silicon probes.

Aβ17−40 and Aβ40 both aggregated as expected and showed substantial toxicity. While there were some differences in aggregation between Aβ17−40 and Aβ40 after 6 and 12 days as measured by ThT, there was no difference in toxicity of the samples at any of the time points tested. We did not observe an increase in aggregation of Aβ17−40 compared to Aβ40 as previously reported (*61*) except for a small increase after 6 days. When either the two-peptide mixture, Aβ17−40 and Aβ1−40, or the three-peptide mixture of Aβ1−16, Aβ17−40, and Aβ1−40 were co-incubated, there was a substantial increase in aggregation after 12 and 18 days, but no corresponding increase in

Table 1: Height Distribution Analysis (%) of Aggregates Obtained by AFM Images of Samples of Aβ40 Alone and Co-incubated with hk14 or c23.5 for 18 days at 37 °C

	molecule height (nm)					
sample	0−1	1−2	2−3	3−4	4−5	5−6
Aβ40 alone	95.6	3.4	0.4	0.2	0.1	0.1
Aβ40 with hk14	89.7	6.6	1.7	0.7	0.3	0.3
Aβ40 with c23.5	97.3	1.7	0.5	0.2	0.1	0.1

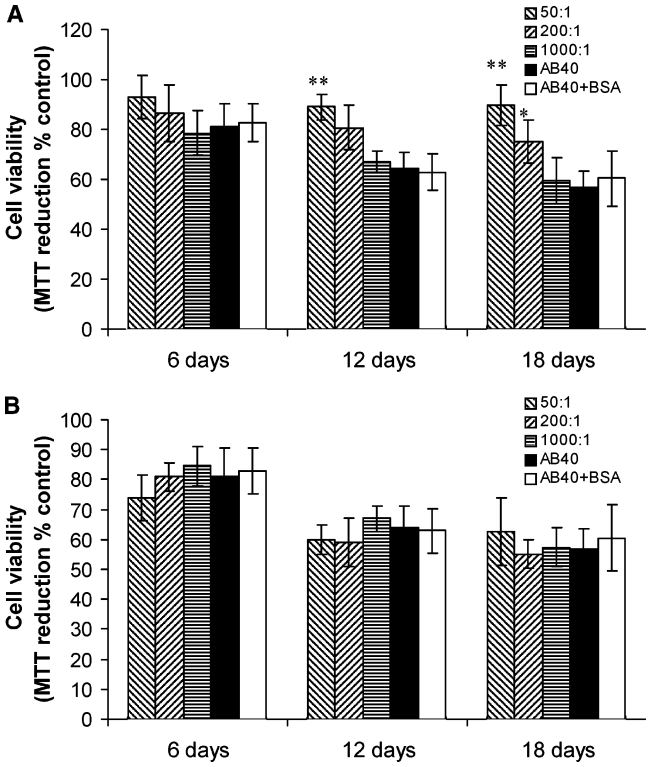

FIGURE 3: Comparison of toxicity of Aβ40 and its mixture with hk14 or c23.5. Eight micromolar Aβ40 with or without different concentrations of hk14 (A) or c23.5 (B) was incubated at 37 °C for 6, 12, 18 days. Ten microliters of sample was added to wells of 96-well plates. The final concentration of Aβ40 in all wells was 400 nM. Data shown are expressed as percentage of control values (one control for each time point) from three independent experiments with each experimental value being the average of six trials. Standard errors of the mean are shown for each sample as bars above the mean (∗, $P < 0.05$; ∗∗, $P < 0.01$ compared to Aβ40 alone).

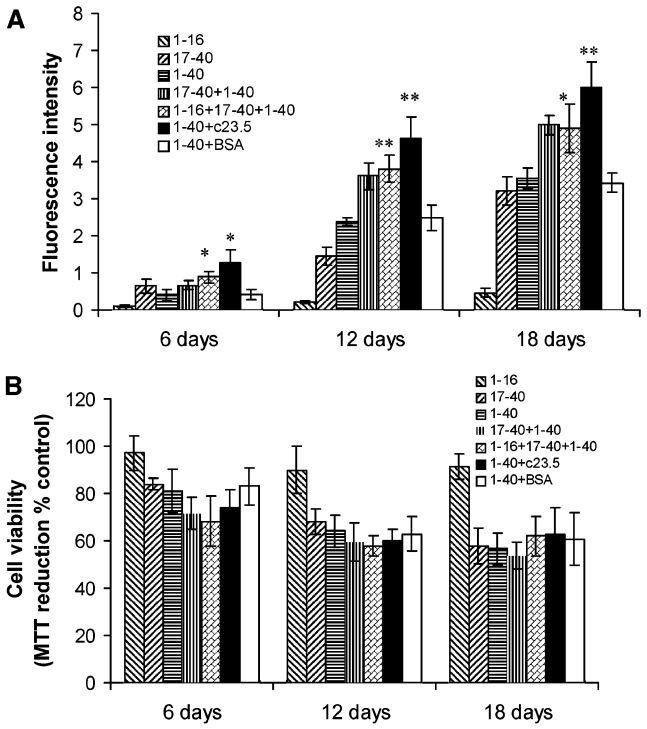

FIGURE 4: Aggregation and cytotoxicity of degradation products of Aβ40 by c23.5. The kinetics of fibril formation was monitored by ThT fluorescence for the samples containing different Aβ peptides (A). The samples were incubated at 37 °C, and 30 μL of samples was removed to 2 mL of 5 μM ThT after 6, 12, and 18 days. Fluorescence intensity was measured at excitation wavelength 450 nm and emission wavelength 482 nm. The experiments were performed in triplicate (∗, $P < 0.05$; ∗∗, $P < 0.01$ compared to Aβ40 alone). The cytotoxicity of the different Aβ peptides samples when incubated for 6, 12, and 18 days was measured using an MTT assay (B). Ten microliters of sample was added to wells of 96-well plates. The final concentration of Aβ40 and total protein of other peptide mixtures in all wells was 400 nM, except for the tripeptide mixture where it was 600 nM. Data shown are expressed as percentage of control values from three independent experiments with each experimental value being the average of six trials.

cytotoxicity; the three-peptide mixture actually showed a decrease in toxicity. The three-peptide mixture comes close to replicating the aggregation results observed when Aβ40 is incubated with c23.5 and almost exactly duplicates the cytotoxicity studies. Addition of a nonspecific protein, BSA, had no affect on aggregation or toxicity compared to Aβ40 alone.

DISCUSSION

The hydrophobic carboxyl terminal region of Aβ is a principal force driving the protein to aggregate (*62*). The longer carboxyl terminal form of the peptide (42 vs 40) favors aggregation (*63*, *64*), as do deletions in the more hydrophilic amino terminal region (*61*, *65*). The amino terminal deleted peptide, Aβ17−40/42 (also referred to as P3) is very insoluble and will form cytotoxic aggregates that may contribute to the neuronal apoptotic characteristic of AD (*66*, *67*). Here we provide further evidence that Aβ17−40 not only aggregates readily but also promotes aggregation when mixed with Aβ40, possibly by providing nucleation sites as previously suggested (*61*). When Aβ40 peptide is incubated with the light-chain fragment c23.5, which we have previously shown to possess α-secretase-like activity toward Aβ40 (*52*), aggregation of Aβ40 is stimulated since the less soluble 17−40 fragment is generated. Similar α-secretase-like cleavage of Aβ by insulin-degrading enzyme (IDE) was previously shown to also enhance oligomerization of Aβ at physiological concentrations in vitro due to the formation of more hydrophobic truncated N-terminal fragments (*49*).

β-secretase, or BACE (β-site APP-cleaving enzyme), is an aspartyl protease that cleaves membrane-bound APP releasing a soluble protein (β-APPs) and generating the N-terminal fragment of Aβ on a 99-residue membrane-bound C-terminal fragment (C99) (*68*, *69*). APP can alternatively be cleaved by α-secretase, which cleaves inside the Aβ region (Lys^{16}-Leu^{17}) to produce α-APPs and a slightly shorter membrane-bound C-terminal fragment (C83) (*39*, *70*). Both C99 and C83 are substrates for γ-secretase (*43*), which can

cleave the intermembrane region of APP to produce the 4-kD Aβ peptide from C99 and the 3-kD peptide (Aβ17−40/42) called P3 from C83. Previous studies have shown that stimulating α-secretase-like cleavage of APP by promoting protein kinase C activity leads to a significant decrease in Aβ formation (*71*−*74*). Since α-secretase cleavage of APP prevents formation of Aβ but increases production of the P3 fragment, increasing α-secretase activity will decrease soluble Aβ levels through two different mechanisms: (1) directly decreasing Aβ production and (2) increasing aggregegation of existing Aβ 40/42 by increasing P3 levels. Our results show that addition of P3 can significantly increase Aβ aggregation in vitro; however, there is no corresponding increase in cytotoxicity. These results indicate that if promoting α-secretase cleavage is to be used as a potential therapeutic approach for treating AD, it may also be beneficial to simultaneously decrease γ-secretase activity. There is still considerable confusion as to whether α-secretase levels play a role in the progression of AD as one study indicated that 80% of samples from AD patients showed decreased α-secretase levels (*75*), while a second report indicated that mRNA levels of a suggested α-secretase candidate ADAM 10 (disintegrin and metalloproteinase 10) was 2-fold higher in AD patients (*76*). Promotion of α-secretase activity toward APP by addition of protein kinase C (PKC) reduced formation of Aβ (*77*).

The p3 fragment, Aβ17−40/42, may play a role in the progression of AD as it has been identified in diffuse plaques of AD patients (*78*), is neurotoxic (*61*), and Aβ17−42 and Aβ1−42 both induce cell death via a similar Fas-like/caspase-8 activation pathway (*67*, *79*). Here we add to these results by showing that Aβ17−40 when added directly to Aβ1−40 or when formed by α-secretase like proteolytic hydrolysis of Aβ1−40, substantially promotes aggregation of Aβ1−40, but does not increase cytotoxicity toward SH-SY5Y cells, even despite the formation of extensive fibrillar structures. These results suggest that Aβ17−40, while it aggregates more quickly than the parent peptide, favors formation of less toxic aggregate morphologies consistent with previous reports showing that enhanced aggregation of Aβ40 did not correlate with increased toxicity to cells (*80*). Many different studies have indicated that the toxicity of Aβ may be due to intermediate structures in the aggregation pathway toward formation of insoluble fibrils, rather than the insoluble fibrils themselves (*10*, *81*, *82*). Apparently Aβ17−40 when mixed with Aβ40 favors formation of a less toxic aggregate morphology.

Our results also show that co-incubation of Aβ40 with hk14 alters aggregation, forming a higher percentage of larger oligomers and extensive very thin filaments. When incubated alone, hk14 also formed oligomers and filaments as indicated by ThT staining and AFM images; however, it showed no toxicity to SH-SY5Y (data not shown). Therefore the low ThT values observed with the hk14/Aβ40 sample is attributable to subtraction of the high fluorescence background of hk14 alone. The very thin filaments observed in Figure 2B may also be partly due to aggregation of hk14; however, the same thin filaments are observed even when Aβ40 is incubated with 2-fold lower concentrations of hk14 (data not shown). The reduced toxicity of Aβ40 when co-incubated with hk14 may be attributable to proteolytic degradation from the carboxyl terminus or to an altered aggregate morphology caused by co-aggregation with hk14 or a combination of both mechanisms.

The amyloid cascade hypothesis (*15*), while still unproven, is a central tenet for AD therapeutic strategies aimed at reducing soluble or aggregated Aβ levels. Such strategies include active and passive immunization approaches to clear soluble and fibrillar Aβ, inhibition of Aβ production by inhibiting β- and γ-secretases, and increasing proteolytic degradation of Aβ. Active and passive immunization protocols can reduce amyloid deposits and decrease soluble Aβ levels (*29*, *83*−*85*) and have been shown to reverse memory deficits (*86*−*88*). Unfortunately, active immunization strategies led to clinical signs and symptoms of meningoencephalitis in a small group of AD patients (*89*, *90*). Postmortem examination of immunized animals with vascular amyloid also showed perimicrovascular hemorrhages and inflammation (*91*−*93*). Since AD is in part an inflammatory disease (*94*, *95*) and immunization strategies may perturb the delicate balance in the brain between healthy maintenance and unhealthy inflammation (*96*), a proteolytic therapeutic approach has great promise. Aβ is normally secreted from cells, and in healthy brains, it is readily hydrolyzed before forming aggregates by various enzymes such as neutral endopeptidase (NEP) (*46*), angiotension-converting enzyme (ACE) (*97*), neprilysin (endopeptidase 24.11) (*98*), endothelin-converting enzyme (ECE) (*50*), and insulin-degrading enzyme (IDE) (*48*, *99*). Since these enzymes have other functions, stimulating their activity to decrease Aβ levels may cause other unwanted side effects. Similarly, the proteolytic antibody light chains reported here, particularly hk14, which can alter Aβ aggregation and inhibit toxicity, in their current state may have very broad specificity and would not be suitable for therapeutic applications. However, recombinant proteolytic antibody light chains are readily adaptable to protein engineering techniques that can greatly increase target specificity. For example pairing of a parent proteolytic light chain with a target specific heavy chain increased the specificity of the resulting construct by 6.6-fold (*100*). Affinity maturation techniques have been effectively used to increase the affinity of parent antibody fragments over a 1000-fold, even into the femtomolar range (*101*) and diabody constructs containing a proteolytic antibody fragment along with a high-affinity antigen-specific antibody fragment can also be constructed. Since none of these constructs would contain the Fc fragment of the antibody, the dangers of aggravating an inflammatory response can be alleviated and the smaller size may facilitate transport across the blood−brain barrier. Therefore our current results indicating that proteolytic antibody fragments can potently alter aggregation and decrease cytotoxicity of Aβ hold great promise for the use of recombinant proteolytic antibody constructs as part of a potential noninflammatory therapeutic treatment and prevention of AD.

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