Heptamer Peptide Disassembles Native Amyloid in Human Plasma Through Heat Shock Protein 70

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Abstract

Proteostasis, which includes the repair and disposal of misfolded proteins, depends, in part, on the activity of heat shock proteins (HSPs), a well-known class of chaperone molecules. When this process fails, abnormally folded proteins may accumulate in cells, tissues, and blood. These species are a hallmark of protein aggregation diseases, but also amass during aging, often in the absence of an identified clinical disorder. We report that a neuroprotective cyclic heptapeptide, CHEC-7, which has been applied systemically as a therapeutic in animal neurodegeneration models, disrupts such aggregates and inhibits amyloidogenesis when added in nanomolar concentrations to human plasma. This effect includes aggregates of amyloid beta (A β 1-40, 1-42), prominent features of Alzheimer's disease pathology. The activity of endogenous HSP70, a recently discovered target of the peptide, is required as demonstrated by both antibody blocking and application of pifithrin- μ , an HSP70 inhibitor. CHEC-7 is the first high-affinity compound to stimulate HSP70's disaggregase activity and therefore enable this endogenous mechanism in a human systemic environment, increasing the likelihood of a convenient therapy for protein aggregate disease, including age-related failures of protein repair.

Keywords: heat shock protein 70, CHEC peptide, amyloid, Alzheimer's, aggregates

Introduction

MORE THAN 40 HUMAN diseases result from the deposition of misfolded and aggregated proteins within cells and in extracellular fluids.^{1,2} These disorders are often characterized by the formation of organized and generally intractable clusters and thread-like aggregates, termed amyloid, that can arise from more than 18 normally soluble proteins. They assemble as toxic participants in well-known disorders and throughout the decline of cell function during typical aging.^{3,4}

One therapeutic strategy for these diseases is to search for compounds that stimulate the normal cellular mechanisms for repair and disposal of misfolded proteins—pathways that become overwhelmed during aging and in age-related disorders.^{5–7} This approach usually involves the regulation of one or more heat shock proteins (HSPs), a class of chaperones, of which HSP70 appears to be the most versatile.

HSP70 has been referred to as an “ATP-driven disaggregase machine”^{8–10} because manipulation of its expression or activity consistently disrupts or inhibits misfolding in aggregation models.^{11–13} In addition, the affinity of HSP70 for specific peptides is critical in immune signaling, particularly with re-

gard to cancer biology.^{14,15} Notably, similar properties have been recognized for extracellular and circulating HSP70,^{16,17} raising the possibility that certain aspects of HSP70 function can be targeted by soluble compounds applied systemically.

CHEC peptides (CHEC-7, CHEC-9) are two of three bioactive peptide sequences that have been identified within the N-terminus of a human protein called DSEP or dermcidin (the third peptide is Y-P30).^{18–21} The parent protein and all peptide derivatives promote cell survival in a variety of experimental models. Importantly, CHECs have been administered systemically to increase neuron survival after lesions to the central nervous system, including in autoimmune models; so far, the heptamer and nonamer peptides have demonstrated similar properties.^{22–24}

One explanation for these effects arose from the identification of the peptides as uncompetitive inhibitors of secreted phospholipase A2.²³ More recently, a peptide that contained a sequence identical or nearly identical to those of CHECs was demonstrated to bind specifically to HSP70.²⁵ The investigators who first described this binding suggested that the chaperone accompanied the peptide to prolong its cell survival activity. Our study of this association *in vivo* in the rat frontal cortex and its effects *in vitro* in a synuclein

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aggregation model (related to Parkinson's disease) suggested that the peptide also influences the activity of HSP70. Peptide treatment increased the supply of disaggregation-competent HSP70 monomers in the cytosol of the rat frontal cortex, presumably by potentiating the chaperone's ATPase.^{12,26,27} Oligomer/monomer equilibrium is a well-known mechanism of regulating protein function. For example, peptide regulation of active monomer levels has been reported for another member of the HSP family, BIP (immunoglobulin heavy chain binding protein), also by stimulating its ATPase activity.²⁸

In this study, we tested the possibility that CHEC-7 would dissociate native amyloid aggregates in the complex milieu of human blood plasma. We proceeded directly to this *ex vivo* human model, bypassing popular transgenic mouse models, due to inconsistent effects when applying CHECs to mice (which do not express the parent DSEP/dermcidin protein). Our model also increases the likelihood that the various accessory proteins, including those that might be necessary for HSP70 function (and possibly existing in forms particular to humans), are present and available.^{29,30} In addition, we focused on the effects of the peptide on the aggregates of amyloid beta peptide (A β 1-42) due to its involvement in the pathology of Alzheimer's disease.

Methods

Human platelet-rich plasma

Platelet-rich plasma (PRP) was purchased from BioIVT (Westbury, NY) and collected in ACD anticoagulant, frozen within 1 hour, and supplied in 1-mL aliquots. The samples were stored at -80°C . The rationale for commercial procurement was the company's standardized preparation methods and rigorous screening policy, and to expedite replication of the present findings. Blood was collected from 18 disease-free donors (aged 26–62 years, 10 males and 8

females) and supplied in 7 lots, each of which was pooled from 2 to 4 donors. The blood was pooled randomly from donors of both sexes by the supplier. The lots were tested as per IRB and FDA regulations and were negative for HBsAG, HIV 1/2 Ab, HCV Ab, HIV-1 RNA, HCV RNA, and STS. At least one assay in the analysis was conducted with a representative sample pair of each of the seven lots with consistent results.

The model

A variety of stress-inducing conditions can lead soluble proteins to form amyloid fibrils and other aggregates. We developed our model through an extensive series of pilot experiments in which we attempted to mimic the eventual failure of repair mechanisms due to extended periods of inflammation and related oxidative stress, all of which can lead to the collapse of the stress response.³¹ This procedure included prolonged but mild denaturation steps, including heat, oxidative stress, and turbulence (Fig. 1). Human PRP was chosen as the starting material to generate these aggregates, because it is a rich source of amyloidogenic proteins, including amyloid precursor protein and the A β 1-40 and 1-42 peptides.^{32,33} H $_2$ O $_2$ treatment and agitation occurred in 1-mL vials on an IKA Vibrax VFR rotary shaker (4-mm diameter, 300 rpm). The 3000 *g* spin to remove platelets was for 15 minutes and the final 11,500 $\times g$ spin was for 30 minutes. The final volume of individual samples (Fig. 1) was between 0.35 and 0.7 mL.

Reagents, dose/response, and antibody reactions

CHEC-7 (CHEASQC) was synthesized by Celtic (Nashville, TN) as a linear sequence and crosslinked at 250 μM in 20 mM Tris, pH 7.8. Internal crosslinking was verified by mass spectroscopy initially and by Ellman's reagent in subsequent batches.²⁴ CHEC-7 was used in all experiments

FIG. 1. Cultivation and treatment of plasma aggregates. The study was designed so that treatment pairs were derived from samples of common pooled lots of PRP and subjected to mild oxidation, heat, and shaking. In some experiments, the paired samples were pretreated with an HSP70 antibody (10 $\mu\text{g}/\text{mL}$) or HSP70 inhibitor (2-phenylethanesulfonamide, pifithrin- μ , 200 μM) or appropriate controls for 2 hours and then with ATP (100 μM) and CHEC-7 (final concentration in plasma 50 nM) or vehicle for an additional 4 hours at 37°C with shaking. The platelets were removed, followed by sedimentation of the remaining particulates from the platelet supernatant. The analysis consisted of testing the particles for amyloid formation and further amyloidogenesis by Tht fluorescence assay and quantifying small A β 1-42-immunopositive aggregates in particulate smears. The particulate supernatant was fractionated by ultrafiltration for Western blot to identify soluble A β 1-42 species in the filtrate and to estimate the concentration of small soluble peptides, presumed remnants of disaggregate activity. HSP70, heat shock protein 70; PRP, platelet-rich plasma; Tht, thioflavin-t.

at a final concentration of 50 nM, as determined in previous studies with human cells and plasma and based on dose/response with the thioflavin-t (Tht) amyloidogenesis assay (Fig. 2A). Solutions of ATP, H_2O_2 , Tht, pifithrin- μ , and bovine serum albumin (BSA, all from Sigma) and A β 1-42 peptide ENZO (ALX-151-002) were all prepared on the day of use, diluted in phosphate-buffered saline (PBS, pH 7.2) or DMSO (pifithrin- μ and A β 1-42 peptide). ATP was added to the samples at a final concentration of 100 μ M (roughly the circulating level of the nucleotide), because it is depleted during *ex vivo* handling of blood³⁴ and because it is essential for HSP70 and CHEC activity (Fig. 2A). HSP70/72 was blocked with an affinity-purified polyclonal antibody ADI-SPA-812-D (ENZO) as a pretreatment before CHEC and ATP addition (Fig. 1). Controls were an affinity-purified species-specific control antibody to GAP43 (Acris via On-line Antibodies #ABIN197546). Pifithrin- μ , an inhibitor of peptide binding to HSP70,³⁵ was added to a final concentration of 200 μ M as also determined by an initial dose/response experiment with the Tht solution assay (Fig. 2B). Pifithrin- μ has been widely used also as a p53 inhibitor.³⁶ However, it is expected that these intracellular activities (inhibition of P53's mitochondrial and BAX protein bind-

ing) would be minimal in this model. The final volume of this or any other reagent added to the plasma was 10 μ L or less per 700 μ L PRP.

The monoclonal antibody against human A β 1-42 used in the Western blots and to stain microscopy specimens was obtained from ENZO (ADI-905-804-100). The secondary antibody was donkey anti-mouse, conjugated to Texas Red or HRP (Jackson Laboratories). Antibody staining procedures for the smears (placed on gelatinized slides) were standard as for tissue sections. Following immunostaining, the smears were incubated in the stock Tht solution (0.8 mg/mL) for 2 hours at room temperature or overnight at 4°C.

Aggregation assays, Western blots, ultrafiltration

For Tht fluorescence assay, the pellet from the 11,500 g spin was mixed vigorously in 15 μ L 10 mM Tris by extensive vortexing and scraping, and a smear was prepared from 2.5 μ L of the suspended pellet and stained as described above. The remaining pellet suspension was then diluted so as to provide 3–4 equally diluted samples (each experimental condition) for the Tht solution assay using a 1:40 dilution of the stock solution.^{37–39} The measurements and monitoring were by a Tecan Infinite Pro 200 reader at 440 nm excitation/482 nm emission, indicative of amyloid formation, after subtracting continuous readings from blanks that contained the Tht solution but no protein. The smears were analyzed by experimentally blinded observers on a Leica M205 FA microscope, fully automated for fluorescence microscopy. Histograms of A β particle sizes were generated from fields of densely populated areas of the smears using Fiji (public access) and Excel (Microsoft Office, 2016). Ultrafiltration of soluble PRP fraction was by centrifugation (YM50, YM3; Millipore) and blots were prepared with SDS but without boiling or chemical reduction after loading 2 μ L (diluted to 15 μ L in sample buffer) of plasma. The filters are rough estimates of the molecular weights of retained proteins and were selected to enrich the filtrates with targeted A β peptides. Due to the various denaturing conditions, the peptide concentrations of the 3 kDa filtrate were estimated, based on absorbance at 215 nm as recommended by Luo for such samples⁴⁰ using the extinction coefficient that was reported by Gunn et al.⁴¹ for A β 1-42. These values were used for treated and control comparisons only (Fig. 5).

Data analysis

Based on our experience with human plasma samples, where baseline and response parameters can vary widely even from the same pooled lot of blood, the experiments were planned as paired comparisons. After an initial 14 hours of stress, peptide-treated and control samples (sometimes subdivided for pretreatment or dose/response experiments) were run side by side through all later experimental manipulations and then compared. Sample size estimates (the number of pairs needed) were made with G-Power (version 3.1) for a paired *t* test with estimates of effect size and variance derived from initial dose/response curves (Fig. 2). A total of 17 pairs were used in this study with each pair run independently but as a part of a set of 4–7 total pairs depending on the assay. The effectiveness of sample pairing was determined by significance with Pearson's correlation. Two-tailed analysis

FIG. 2. (A) Dose/response experiments with CHEC-7 using Tht amyloid assay with and without ATP added to the samples. The various treatments were prepared by subdividing a pretreated PRP sample (Fig. 1) and creating curves from the average of triplicate reactions for each concentration. The effective CHEC-7 dose was 50 nM and inclusion of 100 μ M ATP was required. These experiments also suggested that a total of 23–25 hours from initiating the experiments (~4 hours in the assay itself) were adequate to demonstrate significant changes in amyloid formation. (B) The same procedures were applied to test the effective concentration of the HSP70 inhibitor pifithrin- μ (200 μ M).

FIG. 3. Fluorescent micrographs of Tht-stained amyloid (green), immunopositive Aβ1-42 aggregates (red), or both (merged, yellow) in smears of the final precipitate. In CHEC-treated samples (B, D, F), large areas of the smears were occupied by fine particles. In control samples (A, C, E), the same areas were characterized by a majority of fibrils, larger clusters, or both. (G). Automated counting of Aβ1-42 containing particles ($\leq 1\mu^2$) in six sample pairs revealed an (two- to sixfold) increase in these particles in peptide-treated samples. $p=0.001$, paired t test; Bars are $100\mu\text{m}$.

was used in all comparisons. Some of the comparisons are presented as fold differences (ratios) for clarity and to emphasize the extent of differences observed. If ratios are presented, the statistical tests outlined in the legends were run with the raw data before calculating the ratios. All comparisons were via SPSS statistics.

Results

Aggregate morphology

Tht staining for amyloid and specific immunostaining for Aβ1-42 in smears of the final particulate fraction revealed the same types of profiles that have been described by other investigators for synthetic and native amyloid preparations^{39,42,43} (Figs. 3, 4). These properties include long continuous and short irregular fibrils, decorated linearly with areas of accumulated stain, and spheroid particulate or globular aggregates that varied greatly in size. The latter was the most predominant feature. Many of the aggregates might have associated during the centrifugation steps; thus, to directly compare aggregation activity between samples, we quantified the smallest of Aβ1-42 immunostained aggregates that were amenable to automated quantification (which included particles between $0.8\mu^2$ to $1\mu^2$) in micrographs of the smear (Fig. 3). The effects of the peptide were dramatic. Samples from CHEC-treated PRP contained the vast majority of these small particles in the sample pairs (two- to sixfold excess, Fig. 3G).

CHEC-7's disaggregase activity depends on HSP70 activity

To further test CHEC-7-stimulated disaggregation in these samples and generalize the results to other potentially amyloidogenic proteins, the samples were analyzed with a Tht solution assay, which provides measurable fluorescent signals specific to amyloid formation (Table 1). In these experiments, paired samples were further subdivided for treatment with a polyclonal antibody to HSP70 (used in previous studies to immune precipitate the chaperone¹²) or a species-appropriate control antibody before peptide treat-

ment. The fluorescence values, indicative of existing amyloids and some *de novo* amyloidogenesis occurring during the assay, were decreased significantly by treatment with peptide after the plasma was incubated with the control antibody but not after incubation with the HSP70 antibody (Table 1). This dependence on HSP70 was also tested in samples pretreated with pifithrin- μ ($200\mu\text{M}$, see Fig. 2 for dose/response), an inhibitor of HSP70 substrate binding.³⁵

FIG. 4. CHEC-7 effects on amyloid aggregation in a concentrated albumin solution. Albumin (60mg/mL) containing 0.01% Aβ1-42 peptide was processed using the same steps as for PRP, including CHEC-7 (or vehicle) and ATP addition. Regardless of treatment, the extracted sediment showed elaborate fibrils and aggregates containing Aβ1-42 and other amyloid clusters, the latter presumably formed from albumin (green = Tht, red = Aβ1-42, yellow/orange = both; bar is $100\mu\text{m}$). The inset shows the mean and SEM for the Tht solution assay of three samples for each condition. There were no measurable differences between CHEC-treated and control samples.

TABLE 1. AMYLOID FLUORESCENCE (RFU)

Pair	ConAb+C7	HSP70 Ab+C7	% Diff
1	4872.8	8026.2	60.7
2	3775.9	6567.6	57.5
3	1716.3	6128.2	28.0
4	2552.0	3184.6	80.1
5	3229.3	5976.7	54.0
6	3199.6	5999.4	53.3
	<i>DMSO+C7</i>	<i>Pifithrin μ+C7</i>	
7	1999.8	3554.8	56.3
8	1951.8	3817.8	51.1
9	1656.5	4088.5	40.5
10	1066.3	2624.8	40.6

Thioflavin-t fluorescence measurements following 50 nM CHEC-7 treatment of plasma samples with and without pretreatment with HSP70 antibody or HSP70 substrate binding inhibitor. Both procedures abrogated CHEC-7 reductions in amyloid formation (50 nM C7+Control Ab vs. 50 nM C7+HSP70 Ab, $p=0.003$; DMSO vehicle vs. pifithrin- μ $p=0.003$). Test of effectiveness of pairing: (Pearson correlation=0.822, $p=0.01$). Additional nonsignificant comparisons not represented in the table: 50 nM C7+HSP70 Ab vs. 0 nM+HSP70 Ab, $p=0.145$; 50 nM C7+HSP70 Ab vs. 0 nM+Con Ab, $p=0.955$. Comparisons by paired t test with Bonferroni correction where required.

HSP, heat shock protein; RFU, relative fluorescent units.

The results were consistent with the antibody blocking experiments (Pairs 7–10, Table 1).

Direct CHEC-7 effects on amyloid aggregates

Next, we investigated the possibility that there were direct peptide effects on the extent of aggregation. Such direct effects have been demonstrated for beta-sheet breaker peptides and the N1 fragment of the prion protein, although these were applied at a much higher concentration than that used for CHEC-7.^{44,45} We therefore spiked a concentrated albumin solution (at a similar total protein concentration as plasma) with A β 1-42 (0.01%) monomers and subjected the mixture to the same conditions as for PRP, including the addition of ATP and CHEC-7 (or vehicle). In these cases, sheets and dense fibrils were the predominant aggregate form of A β 1-42, whereas Tht-positive profiles (presumably albumin) formed the familiar clusters (Fig. 4). There was no difference between the CHEC-treated and control samples, including after measurements by the Tht assay (Fig. 4, inset). Therefore, direct effects of the peptide on aggregate formation were undetectable with these procedures.

Soluble remnants of aggregate decomposition

We also examined the soluble fractions of these samples to determine whether there were changes in the composition of the dissolved protein and peptides. Attempts to quantify specific species in Western blots were met with considerable variability, likely due to the nature of plasma and the unpredictable behavior of abundant “sticky” proteins such as albumin and immunoglobulins on polyacrylamide gels, especially under present conditions. However, ultrafiltration of the samples through a 50 kDa MWCO filter allowed us to identify a 54–56 kDa A β 1-42-immunoreactive band in the filtrate along with an undifferentiated trail of smaller im-

munopositive A β peptides (Fig. 4). The band is most likely A β 56*, a stable aggregate that has also been described in human cerebral spinal fluid.⁴⁶ Differences in the apparent levels of this species in sample pairs were not apparent. The relationship of A β 56* to A β toxicity, or whether it is merely a consistent artifact of sample preparation, is not settled so it may be that this aggregate is not targeted by HSP70. In any case, its identification helped to support the validity of the present model since this band commonly appears in other native preparations.

If soluble oligomers are also disassembled after CHEC-7 treatment, we still had to track down any soluble remnants of the disaggregase activity since the usual routes of disposal of potentially toxic amyloid-forming peptides, as well as the other products of the circulating proteasome/ubiquitin system,⁴⁷ are unavailable in this model. For these experiments, the soluble fraction was therefore enriched with small peptides by passing the supernatant through a 3 kDa MWCO filter. The concentration of these peptides was uniformly higher in the 3 kDa filtrate of the CHEC-7-treated samples (pairs 11–17, Fig. 5), suggesting the exaggerated disaggregase activity extended to the soluble aggregates.

FIG. 5. Characterization of soluble peptide remnants of amyloid cultivation and treatment. (A) Representative Western blot of the 50 kDa MWCO filtrate showed a prominent 54–56 kDa band immunopositive for A β 1-42 and identified by others as a soluble oligomer found in native preparations. An indistinct immunopositive trail is also found in the lower molecular weight regions of the blots. CHEC-7 and control samples showed the same pattern with no consistent quantifiable differences in the blots. (B) A 3 kDa MWCO filtrate of the soluble fraction in seven additional pairs of treated and control samples showed a 1.35–5.8-fold increase in the small peptide concentration after CHEC-7 treatment ($p=0.047$, paired t test).

Discussion

The results suggest that extracellular HSP70 activity can be stimulated by the CHEC-7 peptide to disaggregate amyloid in human plasma. Although our model attempted to mimic the “chaperone overload” and subsequent amyloid accumulation suggested to occur in aging and age-related disease, the plasma was collected from healthy relatively young individuals. It is possible that the disaggregase machinery is not simply overloaded in aged or diseased individuals but might also be defective. Therefore, the extent to which the CHEC peptide may be able to delay or prevent the pathologies associated with excessive protein misfolding and aggregation would depend on normal disaggregase mechanisms still in operation.

The remnants of this exaggerated disaggregase activity appeared most consistently in a soluble peptide fraction of the treated plasma consistent with the idea that soluble oligomers (in the case of Ab, now believed to be the most toxic) may be supplied by degradation of amyloid fibrils.⁴⁸ The present results were obtained by measuring a single small peptide fraction and the variability in the data is evident (Fig. 5). It is expected that in the present pooled samples, just as in samples from individual donors, the size and composition of the different remnant multimer pools are likely to be variable and depend on several factors. Therefore, the molecular weight cutoffs we used to isolate the remaining fragments may not have been ideal in every case. In future studies, application of size exclusion methods to individuals or to more specific pools of samples (by age, disease state, or gender) may be more informative.

The relationship of the present disaggregase activities to the anti-inflammatory properties of CHECs, specifically the inhibition of sPLA2 described previously,²³ is not apparent from this study. It has been suggested that HSP70 also influences sPLA2 oligomerization, but the relationship of this process to enzyme activity is unclear.⁴⁹ Other studies suggest that phospholipases A2, including sPLA2, are worthwhile targets for aggregate neurodegenerative disorders such as Alzheimer's disease based on properties of the enzymes at cell membranes, including the production of several proinflammatory lipids.^{50,51} The peptide sequence may also provide clues as to the molecular mechanisms involved in different CHEC activities. It is possible the peptide targets both phospholipases to inhibit the A2 enzyme and also circulating HSP70 to enhance the chaperone's ATPase function. These inhibitory and “mimetic” properties are both common features of peptide fragments and may be based simply on a phospholipase/phosphatase active site sequence motif possessed by the CHECs (CHEXXQC).¹² Thus, the catalytic histidine- and acidic metal-binding residues, flanked by stabilizing crosslinked cysteines, (all elements of the so-called catalytic triad) might constitute the basis for both actions of these peptides. Whatever the case, it is not surprising that anti-inflammatory/antioxidant agents also possess disaggregase properties as demonstrated for both natural compounds and anti-inflammatory drugs.^{52,53}

The focus of this report is the potential of CHEC-7 to stimulate natural disaggregation mechanisms via human HSP70 and the demonstration that these mechanisms are present and accessible in human plasma. The effects of CHEC-7 on cerebral amyloid deposits are unknown at

present. Importantly, a previous report suggests the nonamer CHEC peptide targets HSP70 in cytosolic preparations of the rat frontal cortex. It is likely that the heptamer CHEC peptide also has this property but this suggestion needs to be tested experimentally. Nonetheless, amyloidosis in the blood leads to serious disorders in this and other organs.⁴³ CHEC-7's ability to disperse these aggregates in human plasma may represent a first step in designing strategies to limit these failures of proteostasis as occurring in aggregate disease and typical of aged individuals.

Acknowledgments

This work was supported by the Pennsylvania Department of Health, Commonwealth Universal Research Enhancement Program, and NS055976 from National Institutes of Health. We are grateful to the Spinal Cord Research Center at Drexel University for shared equipment and facilities.

Authors' Contributions

All authors were involved in the planning of the experiments, including design of the aggregation model, and all critically read the article. T.J.C. wrote the original draft. T.J.C., T.C., and L.Y. conducted the assays and data analysis.

Author Disclosure Statement

Three of the authors (T.J.C., J.G., L.Y.), along with Drexel University, hold a patent on CHEC-7, the peptide used in these studies (US No. 8,106,019). There is no commercial licensee of this patent and no author has any affiliation or relationship with any such for-profit entity.

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Received: December 31, 2017

Accepted: April 13, 2018