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Extracellular chaperones modulate the effects of Alzheimer's patient cerebrospinal fluid on A beta(1-42) toxicity and uptake

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Abstract

Alzheimer's disease is characterised by the inappropriate death of brain cells and accumulation of the A beta peptide in the brain. Thus, it is possible that there are fundamental differences between Alzheimer's disease patients and healthy individuals in their abilities to clear A beta from brain fluid and to protect neurons from A beta toxicity. In the present study, we examined (1) the cytotoxicity of Alzheimer's disease cerebrospinal fluid (CSF) compared to control CSF, (2) the ability of Alzheimer's disease and control CSF to protect cells from A beta toxicity and to promote cell-mediated clearance of A beta and lastly (3) the effects of extracellular chaperones, normally found in CSF, on these processes. We show that the Alzheimer's disease CSF samples tested were more toxic to cultured neuroblastoma cells than normal CSF. In addition, the Alzheimer's disease CSF samples tested were less able to protect cells from A beta-induced toxicity and less efficient at promoting macrophage-like cell uptake when compared to normal CSF. The addition of physiologically relevant concentrations of the extracellular chaperones, clusterin, haptoglobin and alpha(2)-macroglobulin into CSF protected neuroblastoma cells from II1-422 toxicity and promoted II1-422 uptake in macrophage-like cells. These results suggest that extracellular chaperones are an important element of a system of extracellular protein folding quality control that protects against A beta toxicity and accumulation.

Keywords

beta, extracellular, uptake, chaperones, modulate, effects, alzheimer, patient, cerebrospinal, fluid, 1, 42, toxicity, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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SHORT COMMUNICATION

Extracellular chaperones modulate the effects of Alzheimer's patient cerebrospinal fluid on $A\beta_{1\text{-}42}$ toxicity and uptake

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Abstract Alzheimer's disease is characterised by the inappropriate death of brain cells and accumulation of the Aβ peptide in the brain. Thus, it is possible that there are fundamental differences between Alzheimer's disease patients and healthy individuals in their abilities to clear Aβ from brain fluid and to protect neurons from Aβ toxicity. In the present study, we examined (1) the cytotoxicity of Alzheimer's disease cerebrospinal fluid (CSF) compared to control CSF, (2) the ability of Alzheimer's disease and control CSF to protect cells from Aβ toxicity and to promote cell-mediated clearance of Aβ and lastly (3) the effects of extracellular chaperones, normally found in CSF, on these processes. We show that the Alzheimer's disease CSF samples tested were more toxic to cultured neuroblastoma cells than normal CSF. In addition, the Alzheimer's disease CSF samples tested were less able to protect cells from Aβ-induced toxicity and less efficient at promoting macrophage-like cell uptake when compared to normal CSF. The addition of physiologically relevant concentrations of the extracellular chaperones, clusterin, haptoglobin and α_2-macroglobulin into CSF protected neuroblastoma cells from $A\beta_{1\text{-}42}$ toxicity and promoted $A\beta_{1\text{-}42}$ uptake in macrophage-like cells. These results suggest that extracellular chaperones are an important element of a system of extracellular protein folding quality control that protects against Aβ toxicity and accumulation.

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Keywords Aβ · Alzheimer's disease · CSF · Extracellular chaperones

Abbreviations

ECs	Extracellular chaperones
BSA	Bovine serum albumin
LRP	Low density lipoprotein receptor related protein
α_2m	α_2-macroglobulin
Hp	Haptoglobin
Aβ	Amyloid beta peptide
QC	Quality control
CSF	Cerebrospinal fluid
ALS	Amyotrophic lateral sclerosis

Introduction

Aβ is the major constituent in extracellular plaques characteristic of Alzheimer's disease and its increased expression is thought to be the triggering event leading to disease pathology (Silvestrelli et al. 2006). This is supported by the fact that, in all cases of early-onset familial Alzheimer's disease where a genetic mutation has been identified, the defective gene causes an increase in production of the more toxic processing variant $A\beta_{1\text{–}42}$ (Mattson 2004; Iwata et al. 2005). The actual mechanism by which Aβ is toxic is unclear; there are several hypotheses. Aβ aggregates, such as mature fibrils (Mattson 2004) and soluble oligomers (Klein 2002), are toxic to cells *in vitro* and are thought to cause pathology in humans

(Gong et al. 2003). Aβ has also been linked to other toxic pathways involving metal ions (e.g. Fe^{2+} and Cu^{2+}) and their subsequent generation of free radicals (Maynard et al. 2005). Aβ is also thought to trigger an inflammatory response in the brain that may underpin disease pathology (Silvestrelli et al. 2006) and be involved in cerebral vascular dysfunction (Greenberg et al. 1996). Regardless of the route of toxicity, it seems that even small increases in soluble $A\beta_{1–42}$ (even as small as a 1.5-fold increase) can result in the appearance of aggressive presenile Aβ pathology (Iwata et al. 2005).

Alzheimer's disease is included in a group of disorders that are associated with inappropriate protein deposits, collectively known as protein deposition disorders. These include Parkinson's disease, amyotrophic lateral sclerosis and Huntington's disease which are associated with deposits of α-synuclein, Cu/Zn superoxide dismutase and huntingtin, respectively. It has been postulated that protein deposits arise when the normally efficient protein folding quality control system is overwhelmed (Muchowski and Wacker 2005; Wilson et al. 2008). However, once in the extracellular space, Aβ is no longer under the surveillance of the intracellular protein folding quality control (QC) system. Recently, it was proposed that an analogous extracellular protein folding QC system exists that recognises non-native proteins and promotes their removal from the extracellular fluid via receptor-mediated endocytosis (Yerbury et al. 2005a, b). It has been shown that clusterin, a ubiquitous and highly conserved secreted protein found in human blood, is a potent extracellular chaperone (Humphreys et al. 1999; Poon et al. 2000; Poon et al. 2002). More recently, both haptoglobin (Hp; previously best known for its high affinity binding to haemoglobin; Yerbury et al. 2005a,b) and α_2-macroglobulin (α_2m; previously best known as an inhibitor of many proteases; French et al. 2008) have been shown to possess potent chaperone actions similar to clusterin. All three of these known extracellular chaperones can be detected co-localised with "plaques" in Alzheimer's disease (Powers et al. 1981; Strauss et al. 1992; Kida et al. 1995). The reason for their presence there is unknown but it is likely that their chaperone action has been overwhelmed and that they have consequently been co-deposited with Aβ (Yerbury et al. 2007). In addition, clusterin, Hp and α_2m have already been shown to inhibit amyloid fibril formation by Aβ *in vitro* (Hughes et al. 1998; Yerbury et al. 2007; 2009). Clusterin and α_2m have previously been shown to complex with Aβ *in vitro* and facilitate its transport *via* receptor-mediated endocytosis to lysosomes for degradation (Hammad et al. 1997; Narita et al. 1997). This is consistent with *in vivo* studies in mice which show that α_2m and clusterin increase the rate of Aβ removal from the brain (Shibata et al. 2000) (Bell et al. 2007).

Since it has been proposed that Aβ metabolism in the brain is dysfunctional in Alzheimer's disease (Iwata et al. 2005), we wondered whether there was a fundamental difference between Alzheimer's disease patients and healthy individuals in their ability to promote cellular uptake/removal of Aβ from CSF or to protect neurons from Aβ toxicity. Previous work has shown that normal human CSF inhibits the formation of Aβ amyloid fibrils *in vitro* while Alzheimer's disease patient CSF does not (Ono et al. 2005). In addition, previous studies have shown that CSF from patients suffering other neurodegenerative diseases such as ALS (Couratier et al. 1994) or Parkinson's (Le et al. 1999) is itself toxic to neuronal cells *in vitro*. In the present study, we examined the toxicity of Alzheimer's disease CSF compared to control CSF. In addition, we examined the ability of Alzheimer's disease and control CSF, with or without added extracellular chaperones, to protect cells from Aβ toxicity and to promote cell-mediated clearance of Aβ.

Results

Alzheimer's CSF is toxic to SY5Y cells

CSF from patients with the neurodegenerative diseases ALS and Parkinson's disease is toxic to cells *in vitro* (Couratier et al. 1994; Le et al. 1999). To determine whether Alzheimer's disease CSF is cytotoxic, SH-SY5Y neuroblastoma cells were incubated in Aim V (Invitrogen, Sydney, Australia) supplemented with a range of levels of human normal and Alzheimer's CSF for 48 h (e.g. a mixture of equal volumes of Aim V and CSF is referred to as 50% CSF). All samples were analysed individually and then the results were averaged. All CSF samples were collected and supplied by the National Neural Tissue Resource Centre (Melbourne, Australia); individual donors were confirmed as Alzheimer's or non-Alzheimer's by post mortem examination of brain sections. Alzheimer's CSF was obtained from three males aged 68, 71 and 82 (designated AD 1, 2 and 3, respectively, in Figs. 2 and 4), while non-Alzheimer's CSF was obtained from three males aged 48, 51 and 69 (designated Normal 1, 2 and 3, respectively, in Figs. 2 and 4). CSF from non-symptomatic donor tissue that contained Aβ plaques were excluded from the "Normal" control samples—this had the consequence that exactly age-matched controls were unavailable. Following incubation with CSF, cells were assayed for viability using calcein-AM (Lichtenfels et al. 1994). The calcein fluorescence of cells incubated with 50% (*v*/*v*) normal control CSF was not significantly different to cells incubated without added CSF (Fig. 1, Student's *t* test, $p > 0.05$), indicating that under the conditions tested normal

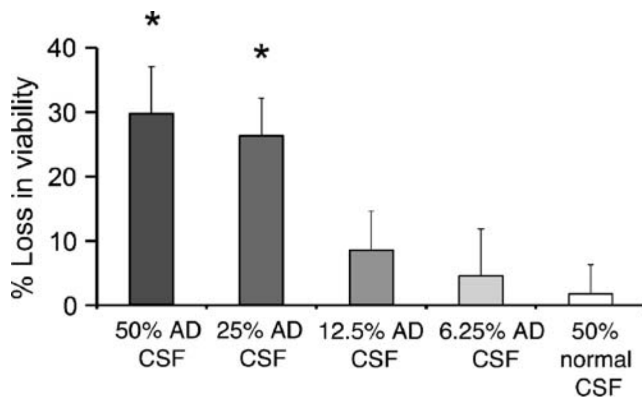

Fig. 1 *AD CSF is toxic to SH-SY5Y cells*. Loss of viability of SH-SY5Y cells, measured as calcein fluorescence and expressed as percentage of untreated control cultures. Cells were cultured for 48 h in FBS-free AIM V medium, with or without various levels of CSF. The % CSF on the *x-axis* corresponds to the amount of CSF present (*v*/*v*) in the media. Calcein-AM was used to measure cell viability. Calcein-AM (1 μM) was added to cells and left to incubate for 30 min before analysing fluorescence using a FLUOstar OPTIMA plate reader with excitation and emission windows of 485±10 nm and 520±10 nm, respectively. Significant differences ($p<0.05$) from the no additives control are indicated by *. The data points shown represent means of all samples from three independent experiments, with 12 samples in total, the *error bars* are SE

CSF was not toxic to SH-SY5Y cells. In contrast, the calcein fluorescence of cells incubated with 50% Alzheimer's CSF was significantly less. The toxicity of Alzheimer's CSF was dose dependent—the level of calcein fluorescence decreased with increasing concentrations of CSF (Fig. 1). The differences between control and Alzheimer's CSF treated wells were statistically significant at both 25% and 50% (*v*/*v*) CSF (Student's *t* test, $p<0.05$). Fluorescence microscopy of calcein-AM stained cells confirmed that there were less viable cells and loss of neurite outgrowth in wells containing 25% and 50% Alzheimer's CSF compared to those incubated with control CSF (data not shown).

Addition of extracellular chaperones protects cells from Alzheimer's CSF toxicity

Immunoblotting analysis of the CSF samples used in this study showed that the levels of all three extracellular chaperones tested (α_2m, haptoglobin and clusterin) were higher in control CSF than in Alzheimer's CSF (Fig. 2a). In addition, a BCA assay indicated that the total protein concentrations in the Alzheimer's CSF samples used in this study were on average lower than that of normal CSF (Fig. 2b). Thus, the difference in extracellular chaperone levels may be due to differences in total protein concentration between control and Alzheimer's CSF, rather than a selective depletion of extracellular chaperones in Alzheimer's CSF (Fig. 2a, b). Regardless, the differences in extracellular chaperone levels prompted us to test whether adding exogenous extracellular chaperones into Alzheimer's CSF would protect cells from its toxicity. Compared to cells incubated with Alzheimer's CSF alone, those incubated with Alzheimer's CSF supplemented with 2 μg/ml clusterin, 2 μg/ml haptoglobin and 4 μg/ml α_2m showed significantly greater viability after 48 h (Fig. 2c; $p<0.05$). In contrast, when the same concentrations of extracellular chaperones were added to normal CSF, this had no effect on the resulting cell viability (Fig. 2c).

Alzheimer's CSF promotes $A\beta_{1\text{-}42}$ toxicity which is modulated by extracellular chaperones

$A\beta_{1\text{-}42}$ was incubated under amyloid forming conditions, as previously reported (Yerbury et al. 2007), and samples removed at 2 h. When incubated with cells in the absence

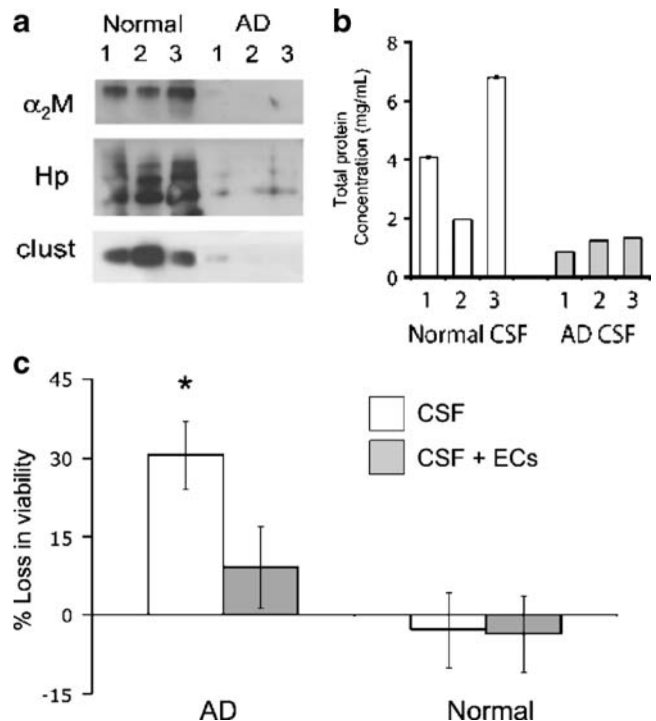

Fig. 2 *Extracellular chaperones protect SH-SY5Y cells from AD CSF toxicity.* **a** Western blot images showing levels of α_2m, Hp and clusterin in 5 μl of CSF. **b** Protein concentration in CSF samples measured using BCA assay. BSA was used to create a standard curve and the assay was conducted as per the manufacturer's instructions. For (**a**) and (**b**), individual CSF samples are labelled *1–3* (see first paragraph in Results section). **c** Loss of viability of SH-SY5Y cells, measured as calcein fluorescence and expressed as percentage of untreated control cultures. Cells were cultured for 48 h in FBS-free AIM V medium, with or without individual CSF samples and/or a cocktail of 2 μg/ml clusterin, 2 μg/ml haptoglobin and 4 μg/ml α_2m. 1 μM Calcein-AM was used to measure cell viability. Significant differences ($p<0.05$) from the control CSF sample are indicated by *. The data points shown represent means of all samples from three independent experiments, with nine samples in total, the *error bars* are SE

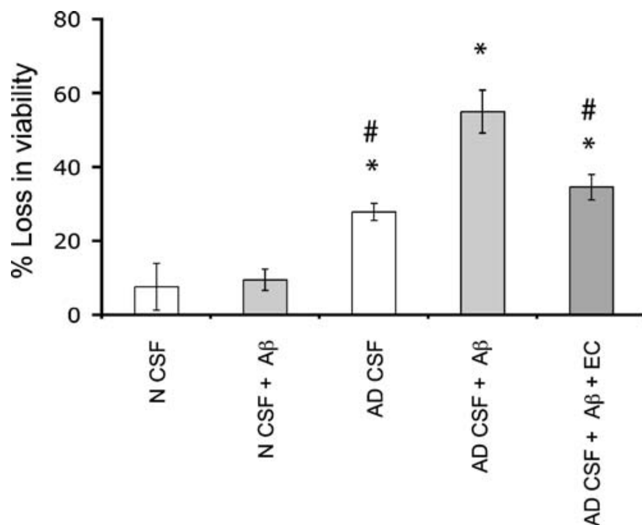

Fig. 3 *Normal CSF and extracellular chaperones protect SH-SY5Y cells from $A\beta_{1-42}$ toxicity.* Loss of viability of SH-SY5Y cells, measured as calcein fluorescence and expressed as percentage of untreated control cultures, incubated for 48 h with $A\beta_{1-42}$ alone or in the presence of CSF with or without added extracellular chaperones (2 μg/ml clusterin, 2 μg/ml haptoglobin and 4 μg/ml α_2m). To induce cytotoxicity, aliquots of $A\beta_{1-42}$ reactions (taken at 2 h) were added to cells alone to give final concentrations of 1 μM. Aggregation of $A\beta_{1-42}$ (10 μM) was initiated by incubation in PBS containing 90 mM $CaCl_2$, 50 mM $MgCl_2$, 100 mM $CuCl_2$, 600 mM glycine, pH 7.5. Significant differences from the normal CSF alone sample are indicated by *. Significant differences from the AD CSF+Aβ sample are indicated by #. The data points shown represent means of all samples from three independent experiments, with nine samples in total, the *error bars* are SE

of CSF, these prefibrillar fractions were toxic to SH-SY5Y cells in a dose-dependent manner (Yerbury et al. 2007). The possibility that CSF could protect cells from toxic $A\beta_{1-42}$ species was examined. When toxic $A\beta_{1-42}$ aggregates were added to cells incubated in 50% (*v*/*v*) normal CSF, there was no significant loss of viability (Fig. 3). In contrast, when the same dose of $A\beta_{1-42}$ aggregates were added to cells incubated with 50% Alzheimer's CSF, a significant loss of viability resulted (Fig. 3, Alzheimer's CSF+Aβ versus N CSF+Aβ; Student's *t* test, $p<0.05$). Addition of extracellular chaperones to cells incubated with Aβ supplemented Alzheimer's CSF provided significant protection (Student's *t* test, $p<0.05$). However, the protection provided by extracellular chaperones did not bring cell viability back to the level of controls (Fig. 3 Alzheimer's CSF+Aβ+extracellular chaperone versus N CSF+Aβ; Student's *t* test, $p<0.05$).

Extracellular chaperones promote macrophage-mediated uptake of $A\beta_{1-42}$ from CSF

Since the metabolism of $A\beta_{1-42}$ in the brain is thought to be defective (Iwata et al. 2005), we examined the possibility that Alzheimer's CSF negatively affected the uptake of $A\beta_{1-42}$ by macrophages. Aβ was added to PMA-differentiated macrophage-like U937 cells incubated with either Alzheimer's or normal CSF (50%) and after 24 h the supernatant was collected and analysed by immunoblotting. In all cases, the supernatant from cells incubated with Alzheimer's CSF contained more $A\beta_{1-42}$ than supernatant from cells incubated with normal CSF (Fig. 4). In two of three cases, there was no remaining Aβ detected in culture supernatants of cells incubated with normal CSF samples (Fig. 4). A large fraction of the $A\beta_{1-42}$ present in supernatants from cultures incubated with Alzheimer's CSF was present as SDS-resistant oligomers. The oligomers ranged from around 10 kDa to just under 25 kDa, corresponding to two to five Aβ monomers (one monomer ~4.5 kDa) (Fig. 4). When compared with corresponding non-extracellular chaperone-supplemented cultures, the addition of extracellular chaperones decreased the amount of $A\beta_{1-42}$ remaining in the supernatant (Fig. 4). Furthermore, for the Alzheimer's CSF supplemented cultures, the addition of extracellular chaper-

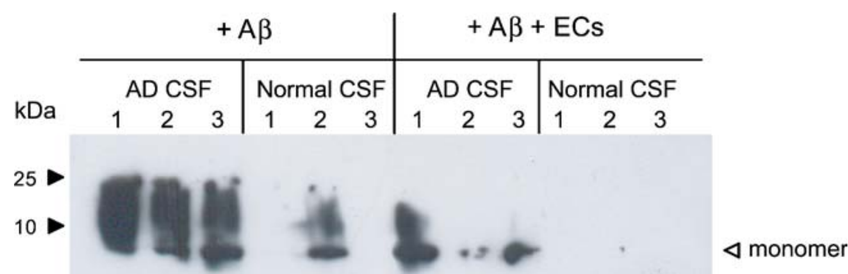

Fig. 4 *AD CSF inhibits $A\beta_{1-42}$ uptake by macrophage-like cells.* Western blot image of $A\beta_{1-42}$ remaining in the CSF containing supernatant after incubation with PMA-differentiated macrophage-like U937 cells. Exogenous $A\beta_{1-42}$ was added to CSF to give a concentration of 1 μM. The CSF was diluted 1:1 in AIM V and added to cells and incubated for 24 h. At the conclusion of the incubation, samples were centrifuged for 10 min at 3,000×*g*. The supernatant was removed and analysed by 15% SDS PAGE and subsequent immunoblotting using WO2 (anti-Aβ monoclonal antibody). Bound specific antibodies were detected with HRP-conjugated sheep anti-mouse Ig antibody followed by enhanced chemiluminescence (ECL) with Supersignal West Pico Chemiluminescent Substrate. Individual CSF samples are labelled *1–3* (see first paragraph in Results section). Results shown are representative of at least two independent experiments

ones reduced the fraction of supernatant Aβ found in SDS-resistant oligomers (Fig. 4).

Discussion

It is thought that an increase in Aβ production and/or a reduced rate of clearance of Aβ from extracellular central nervous system fluids may be contributing factors in the development of Alzheimer's pathology (Iwata et al. 2005). One or more types of soluble Aβ species are likely to be responsible for the neurotoxicity associated with Alzheimer's disease, as the pathology is correlated with elevated levels of Aβ in the brain (Naslund et al. 2000) but does not correlate well with the location of insoluble Aβ plaques (Terry et al. 1991). In the current study, we have shown that Alzheimer's CSF is toxic when incubated with neuroblastoma cells of human origin (SH-SY5Y) (Fig. 1). The toxicity can, at least in part, be suppressed by the addition of extracellular chaperones at concentrations equivalent to those found in normal human CSF. The extracellular chaperones clusterin, haptoglobin and α_2m are present in normal human CSF at 2 μg/ml, 0.5–2 μg/ml and 1–3.6 μg/ml, respectively (Murphy et al. 1988; Sobek and Adam 2003; Biringer et al. 2006). The mechanism by which Alzheimer's CSF is toxic and the means by which extracellular chaperones protect cells from Alzheimer's CSF cytotoxicity is unknown. Extracellular chaperones, such as clusterin, have been shown to protect cells from a range of stresses (Wilson and Easterbrook-Smith 2000). Clusterin and α_2m have both been shown to protect cells from Aβ toxicity (Boggs et al. 1996; Fabrizi et al. 2001) and TNFα-mediated cell death (Humphreys et al. 1997; Arandjelovic et al. 2007). α_2m can also protect cells by binding to and trapping proteases (Ikari et al. 2001). It has been demonstrated that both clusterin and Hp protect cells from oxidative stress-induced toxicity (Schwochau et al. 1998; Melamed-Frank et al. 2001). It is possible that one or more of these potential mechanisms of toxicity are being blocked by the action of the extracellular chaperones. Collectively, this suggests that these extracellular chaperones may play a cytoprotective role in Alzheimer's disease.

We next examined the ability of CSF to protect neuroblastoma cells from the toxicity of added exogenous $A\beta_{1\text{-}42}$. Under the conditions tested, normal CSF protected cells from $A\beta_{1\text{-}42}$ toxicity, while Alzheimer's CSF did not (Fig. 3). This is consistent with the fact that the *in vitro* formation of amyloid fibrils by Aβ is inhibited by normal human CSF but less so by Alzheimer's CSF (Ono et al. 2005). Taken together, these results suggest that, at least in the samples tested, there is a fundamental change in the composition of Alzheimer's CSF that compromises its ability to inhibit the aggregation and toxicity of Aβ. In the current study, this may relate to the differences found in the levels of the extracellular chaperones clusterin, α_2m and Hp. The lower total protein concentration of Alzheimer's CSF may account for the lower concentrations of extracellular chaperones in Alzheimer's CSF found in this study, suggesting that extracellular chaperones are not selectively depleted from this fluid. This is consistent with previous work showing that Alzheimer's disease patient CSF has a lower total protein concentration than normal CSF controls (Morihara et al. 1998). In contrast, previous studies suggested that the concentration of at least one of the extracellular chaperones, clusterin, is not lower in Alzheimer's CSF (Lidstrom et al. 2001; Nilselid et al. 2006). However, while previous studies suggest that clusterin concentration does not differ in spinal fluid of Alzheimer's patients, it is possible that the differences in relative clusterin levels in spinal fluid described in the current study could be attributed to technical differences between the Western blotting technique used here and the ELISA method used previously (Lidstrom et al. 2001; Nilselid et al. 2006). It is also impossible to rule out the possibility that the differences seen in the present study could be due to the small sample size used.

In the current study using Western blotting, we show that the levels of clusterin, α_2m and Hp are lower in samples of Alzheimer's CSF compared to normal CSF. Moreover, addition of exogenous extracellular chaperones into Alzheimer's CSF provided partial protection to neuroblastoma cells from Aβ toxicity. Previous studies have also shown that clusterin and α_2M protect cells in primary rat mixed neuronal cultures from Aβ toxicity (Boggs et al. 1996; Du et al. 1997). Taken together, these results are consistent with the idea that, in the CSF of Alzheimer's patients, lower levels of extracellular chaperones may render the CSF less able to sequester and safely remove Aβ than is the case in healthy individuals. Both α_2m and clusterin have been shown to mediate cellular Aβ uptake via receptor-mediated endocytosis (Hammad et al. 1997; Narita et al. 1997). This is in accordance with the theory that these extracellular chaperones play a role in sequestering and disposing of dangerously hydrophobic proteins or peptides *in vivo* (Yerbury et al. 2005a,b; Wilson et al. 2008). The current study suggests that Alzheimer's CSF is less effective at promoting the cellular uptake of exogenously added Aβ than control CSF and that the addition of extracellular chaperones can promote the uptake of $A\beta_{1\text{-}42}$ by macrophage-like cells. This is consistent with *in vivo* studies that show that the rate of clearance of Aβ from the mouse brain is significantly increased by both α_2m (Shibata et al. 2000) and clusterin (Bell et al. 2007).

In conclusion, the results presented here are consistent with a model in which extracellular chaperones bind Aβ and maintain its solubility, protect cells from its toxicity and

subsequently promote its removal from the brain fluid via receptor-mediated endocytosis. Results from this study suggest that Alzheimer's CSF is less able to protect cells from Aβ toxicity and promote removal of Aβ from CSF. In addition, our results suggest that increasing the concentration of extracellular chaperones in Alzheimer's brain fluid may alleviate Aβ toxicity and promote its removal from the brain. Thus, increasing the concentration of extracellular chaperones in the brain may represent a potential therapeutic strategy for the treatment of Alzheimer's disease.

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