Effect of chloroquine and leupeptin on intracellular accumulation of amyloid-beta (Aβ) 1–42 peptide in a murine N9 microglial cell line

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Abstract Murine N9 microglia accumulated Aβ from media containing 0.67 μM Aβ within 6 h. In N9 and in primary rat microglia, chloroquine, which disrupts lysosomal pH, increased Aβ-induced accumulation of Aβ, particularly Aβ1–42. Leupeptin similarly enhanced Aβ accumulation. The scavenger receptor antagonist fucoidan did not affect acute chloroquine-dependent Aβ1–42 accumulation, demonstrating uptake of non-aggregated Aβ. After prolonged incubations, chloroquine enhanced Aβ multimer (8–12 kDa) accumulation, an effect inhibited by fucoidan. Disruptions of the lysosomal system enhance Aβ and its multimer formation. Despite negligible effects of fucoidan on initial Aβ uptake, chronic exposure inhibits multimer accumulation, demonstrating a role for scavenger receptor in multimer accumulation.

Key words: Alzheimer's disease; βA4 amyloid; Lysosome; Microglia; Chloroquine; Leupeptin

1. Introduction

A pathological hallmark of Alzheimer's disease (AD) is the cerebral deposition of amyloid plaques which arise from the abnormal accumulation of amyloid β-protein (Aβ). Aβ peptides are 40–43 amino acid proteolytic fragments excised from β-amyloid precursor protein (β-APP). Compelling genetic evidence has indicated that over-production of Aβ, especially Aβ1–42, can be an ultimate cause of neurodegeneration. Among cases of early-onset familial Alzheimer's disease (FAD), an autosomal dominant disorder, mutations are found in multiple genes [1,2]. These mutations increase either the production of Aβ or the proportion of the more insoluble Aβ1–42 fragments. In contrast, APP mutations and systemic Aβ over-production are not found in the late-onset, sporadic form of AD (SAD) [1] which accounts for the vast majority (>90%) of AD cases. Therefore, factors such as decreased cellular degradation of Aβ peptide or aggregates, reduced Aβ phagocytosis, and enhanced stabilization of Aβ fibrils may be particularly relevant to Aβ accumulation in SAD. Aβ is secreted by brain cells under normal conditions [3,4], but its level remains low in CSF and plaque-free brains of healthy controls [5]. Therefore, a catabolic process must exist in which protease(s) degrade the peptide to prevent its accumulation. A major difference between an aging AD brain and a healthy counterpart could be defective catabolism which results in Aβ accumulation and deposition.

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Cellular uptake and intracellular degradation are potentially important pathways for the removal of extracellular Aβ. PC12 cells were able to effectively internalize Aβ and accumulate Aβ1–42 specifically for several days [6]. Ida et al. [7] demonstrated the rapid Aβ uptake by human neuroblastoma SY5Y cells which removed the majority of exogenous Aβ by uptake and intracellular degradation rather than by extracellular proteases. However, these cells do not resemble the activated microglia and reactive astrocytes intimately associated with dense core amyloid plaques in AD brain [8]. Both rat microglia and astrocytes can remove Aβ1–42 added to culture medium [9]. After subjecting rat primary microglia to subcellular fractionation, Mentlein et al. [10] recently showed that an Aβ-degrading activity was primarily mediated by a metalloprotease released into the extracellular medium. Other studies have shown that rat microglia are able to accumulate Aβ peptides [11] and aggregates [12], suggesting that intracellular degradation via the endosomal-lysosomal system is important for Aβ removal. Both APP and APP C-terminal fragments are known to be metabolized by the endosomal-lysosomal system [4,13–15]. Furthermore, lysosomal proteases such as cathepsins B (cysteine protease) and D (aspartyl protease) are associated with AD amyloid deposits [16,17]. The presence of these lysosomal enzymes in the amyloid plaques may reflect an attempt to degrade Aβ. To test whether microglia degrade Aβ intracellularly, we examined the accumulation and degradation of added Aβ1–40 and 1–42 in the N9 murine microglia cell line. A significant amount of undegraded Aβ, especially Aβ1–42, was found inside the cells with chloroquine and leupeptin, compounds which inhibit lysosomal enzyme activities.

2. Materials and methods

2.1. Sandwich ELISA for Aβ

Cellular Aβ was quantified with a sandwich ELISA utilizing two Aβ peptide-specific antibodies. Mouse monoclonal 4G8 against Aβ aa 17–24 (Senetek, Maryland Heights, MO) was used as the capture antibody, loaded at a concentration of 3 μg/ml in 0.1 M carbonate buffer, pH 9.6 onto a 96-well plate (Nunc Maxisorp). Blocking was completed with 2% bovine serum albumin (BSA) in Tris-buffered saline (TBS). Processed and neutralized samples were diluted with EC buffer (20 mM Tris, 400 mM NaCl and 2 mM EDTA, pH 7.4 with 1% BSA and 0.05% CHAPS) containing protease inhibitors (20 μg/ml each of pepstatin, aprotinin, leupeptin, and phosphoramidon, 0.5 mM PMSF and 1 mM EGTA) and loaded into the wells. The detector antibody was biotinylated mouse monoclonal 10G4 against Aβ aa 5–13 [18], added at a final dilution of 1:1500 in TBS containing 1% BSA. The reporter system was streptavidin-alkaline phosphatase (Vector Labs, Burlingame, CA) using AttoPhos (JBL, San Luis Obispo, CA) as the substrate. Fluorescence of the AttoPhos product was monitored at an excitation wavelength of 450 nm and an emission

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wavelength of 580 nm with a CytoFluor II plate reader (PerSeptive Biosystems, Bedford, MA). A standard curve over a range of 0.02–10 ng (Fig. 3A) was prepared from Aβ peptide in triplicate and subjected to 4-parameter fit by non-linear regression. Statistical ANOVA was used to evaluate treatment effects and Fisher's projected of least significant difference (PLSD) was used to detect differences between groups (Statview, Abacus, Berkeley, CA).

2.2. Cell culture

N9 microglia (a mouse microglia cell line, a generous gift from Dr. P. Ricciardi-Castagnoli) [19] were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose and supplemented with 10% fetal calf serum, L-glutamine, non-essential amino acids, sodium pyruvate, HEPES buffer, and gentamicin sulfate. Cells were cultured in 75 cm^2 flasks and passaged without trypsin using cell dissociation buffer (Sigma, St. Louis, MO). They were subcultured into 24-well plates at 80% confluency or Nunc 8-chamber Labtek slides at 5000/well 1 day before the experiment. For primary mixed glia culture, cerebral hemispheres from 2 day old Sprague-Dawley rat pups were excised and the meninges removed. The hemispheres were filtered successively through 210, 185, and 136 μm nylon meshes into DMEM with the same supplements described above. The cell suspension was centrifuged and resuspended in media before seeding 10^6 cells to a 75 cm^2 flask. Primary cultures were incubated for 5 days before use.

2.3. Analysis of intracellular Aβ after cellular uptake

Confluent N9 microglia in a 24-well plate were washed once with phosphate-buffered saline (PBS) and once with N2 medium (serum free) before exposure to 300 μl of N2 medium containing 1 μg (0.67 μM) of Aβ, with and without chloroquine or leupeptin. Cells were incubated with the drug(s) for 6 h in the CO_2 incubator with gentle shaking. At the end, the medium was removed and the cells washed twice with PBS before trypsinization which removed cell membrane associated Aβ. After a centrifugation at $1000 \times g$ for 10 min, the cell pellet was washed once with PBS and stored at −20°C until assay of Aβ. On the day of analysis, cell pellets were solubilized in 70% formic acid. Acid extract was neutralized with 70% 0.25 M Tris, pH 8/30% acetonitrile and 5 M NaOH before loading onto the ELISA plate. Total cell protein was measured by the Bio-Rad Dc (Hercules, CA) Lowry assay using BSA as a standard.

2.4. Immunofluorescent labeling of intracellular Aβ

N9 microglia grown on 8-chamber Labtek slides were exposed to 1 μg of Aβ, with or without 20 μM chloroquine, for 6 h. At the end, the cells were washed 3 times with cold PBS and fixed with cold methanol at 4°C for 10 min, then methanol was removed and cells washed 3 times with cold PBS. After blocking with 5% horse serum at 37°C for 1 h, cells were incubated with mouse monoclonal 4G8 (1:100) at RT for 1 h. Bound 1° antibodies were detected with horse anti-mouse IgG (2° antibody, 1:200) coupled to FITC and photographed using a Microphot fluorescence microscope (Nikon).

All images were acquired as TIFF files from a fluorescent microscope via a video camera attached to a Scion frame grabber, which were subsequently analyzed on a Power Center 120 Macintosh compatible computer using a NIH-Image public domain software (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). A custom Pascal macro sub-routine was written to calculate fluorescent intensity relative to background (foreground−background/background) and total % Aβ-ir area.

2.5. Western blotting

Equal amounts of protein from the cell pellets were mixed with Laemmli's sample buffer and heat-denatured before being loaded onto 8–16% Tris Tricine gel with rainbow standards (Amersham, Piscataway, NJ) as molecular weight reference. Separated proteins were blotted onto nitrocellulose membrane and detected with sequential incubation with mouse monoclonal 10G4 1° antibodies (1:750), goat HRP-conjugated anti-mouse IgG 2° antibodies (1:5000) and subsaturating ECL solution (Amersham).

3. Results

3.1. Effects of chloroquine on intracellular Aβ level quantified by sandwich ELISA

As the cells were trypsinized to remove membrane-associated Aβ, any detectable signal in the ELISA must have originated from the intracellular compartment. When N9 microglia were exposed to 1 μg (0.67 μM) of Aβ (1–40 or 1–42)

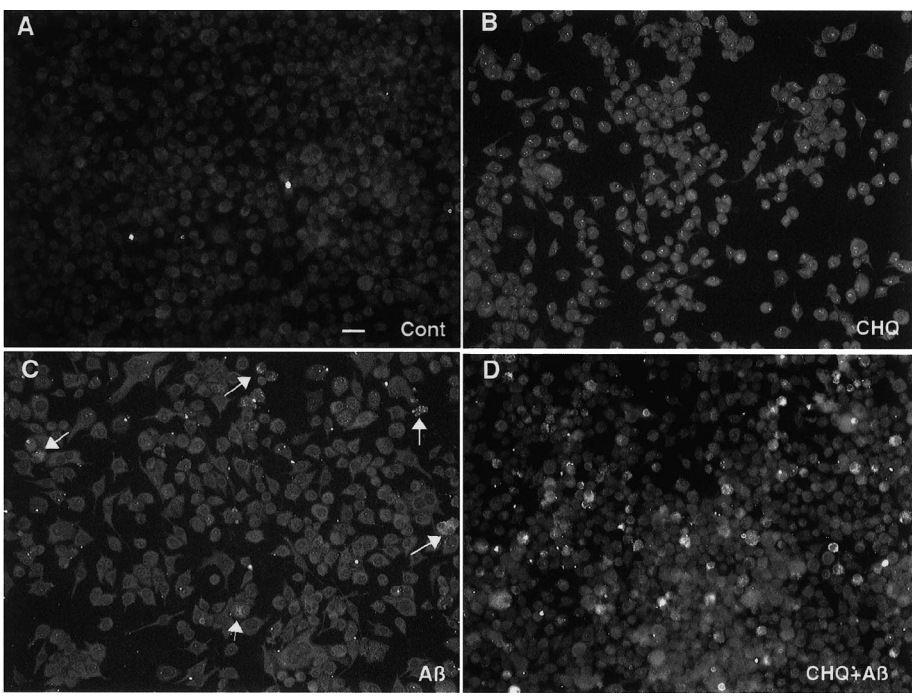

Fig. 1. Immunofluorescent staining of Aβ1–42 with and without chloroquine (CHQ, 20 μM). Cell-associated Aβ was detected by mouse monoclonal 4G8 (1° antibody) followed by horse anti-mouse IgG (2° antibody) coupled to FITC and visualized under the fluorescence microscope. N9 microglia were incubated with (A) vehicle, (B) CHQ, (C) Aβ1–42 (arrows indicate selected isolated cells that are actively taking up Aβ), and (D) CHQ+Aβ1–42. Bar = 80 μm.

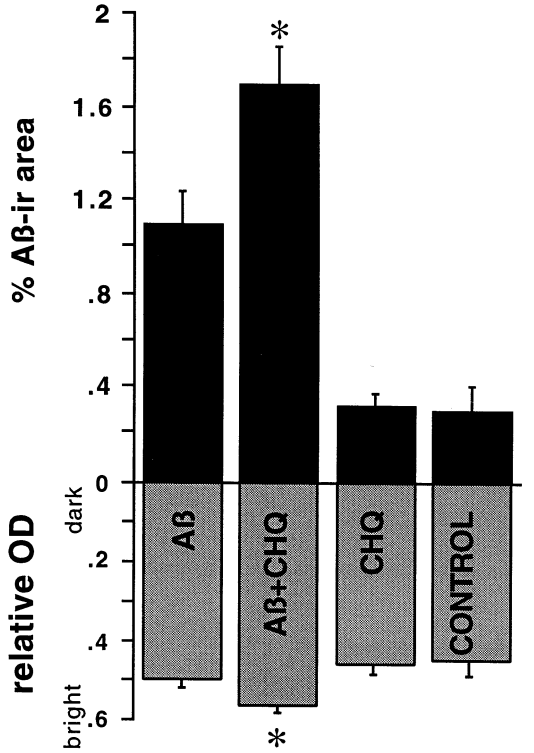

Fig. 2. Image analysis of cells treated with Aβ42 with or without chloroquine (CHQ). Data show % Aβ-ir area (black) and fluorescent intensity (gray) in arbitrary units as relative OD (foreground−background/background). CHQ+Aβ42 showed more area stained as well as higher intensity than Aβ42 alone ($P<0.05$), and values from both these treatments were significantly greater than CHQ or Aβ42 alone ($P<0.05$). CHQ or Aβ42 alone were not significantly different from each other.

alone for 6 h, Aβ1–42 preferentially accumulated intracellularly while the level of Aβ1–40 was non-detectable (Fig. 3B). The intracellular level of undegraded Aβ, especially that of Aβ1–42, increased substantially in the presence of chloroquine, a weak base that disrupts lysosomal functions [20] by dissipating the transmembrane pH gradient (Fig. 3B). Intracellular Aβ1–40 was detected only in the presence of higher chloroquine concentrations (40 and 125 μM), but its level was still lower than that of Aβ1–42. There was no gross loss of viability upon 6 h chloroquine incubation as LDH release was similar in both control and treated cells (data not shown). The chloroquine-dependent accumulation of Aβ1–42 after 6 h in N9 microglia was also seen in primary rat microglia which accumulated 0.2075 ± 0.0275 ng of Aβ1–42/mg protein in the absence of chloroquine and 0.3175 ± 0.012 ng Aβ1–42/mg protein in the presence of 40 μM chloroquine ($P<0.05$, significantly different from Aβ only). Chloroquine alone (without exogenous Aβ) may also enhance accumulation of endogenous Aβ as demonstrated by a consistent, although faint pattern of intracellular Aβ-ir punctate labeling in the Fig. 1B micrograph, a pattern not visualized in other treatment groups. However, quantitative analysis of cells (both N9 and primary rat microglia cells) treated with chloroquine alone (40 or 125 μM) was not sensitive enough to pick up the difference between controls and chloroquine alone. In addition, Aβ-bearing C-terminal fragments of APP are also produced and processed in the endosomal/lysosomal compartment [4]. Thus, chloroquine may cause an accumulation of endogenous C-terminal fragments by interfering with their lysosomal degradation. The sandwich ELISA was specific for Aβ since 10G4 fails to detect APP (data not shown). The absence of any ELISA immunoreactivity in cells treated with chloroquine alone indicated that C-terminal fragments are not detected in ELISA.

3.2. *Effect of chloroquine on cell-associated Aβ revealed by immunofluorescent labeling*

In order to confirm that chloroquine treatment increases cellular Aβ, we measured Aβ-immunoreactive fluorescence (ir) using anti-Aβ (4G8) after 6 h incubation (Fig. 1). No Aβ-ir was observed in control cells with no treatment (Fig. 1A) or in cells untreated with primary antibody (not shown). Chloroquine alone induced a characteristic punctate intracellular Aβ-ir labeling (Fig. 1B), but this did not result in quantitative differences in total fluorescence intensity or % Aβ-ir area (Fig. 2). Aβ1–42 (Fig. 1C) induced what appeared to be clusters of cells with both surface labeling and some perinuclear labeling. Addition of chloroquine to Aβ1–42-treated cells resulted in a dramatic upregulation of Aβ-ir, which was predominantly perinuclear. Addition of Aβ1–40 alone or in conjunction with chloroquine resulted in similar but less pronounced effects compared to Aβ1–42 (not shown).

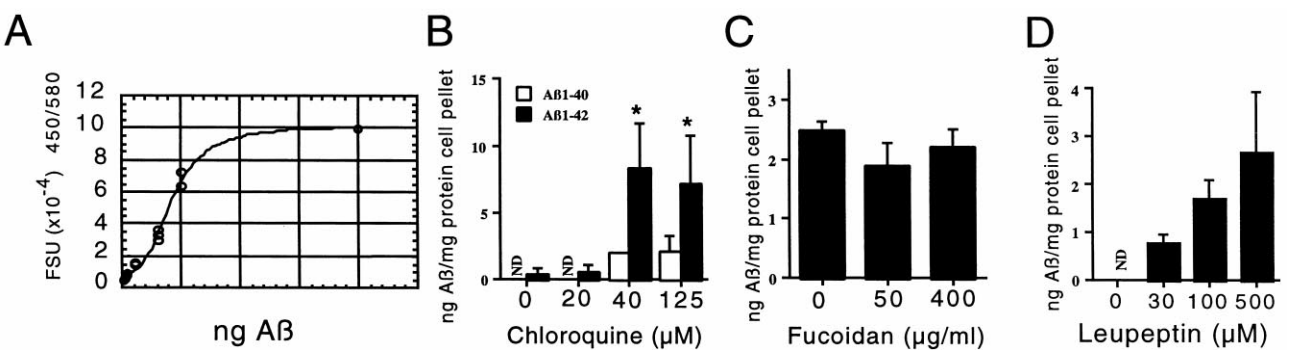

Fig. 3. A: A typical ELISA standard curve with 4-parameter fit by non-linear regression, $r^2 = 0.994$ and the lowest detection limit is 10 pg in our experiments. B–D: N9 microglial cells were treated with 1 μg of Aβ1–40 or 1–42 and cells harvested at 6 h. Cells were harvested and Aβ in the cell pellet measured by sandwich ELISA. B: Dose-dependent effect of chloroquine (CHQ) on intracellular Aβ (1–40 and 1–42) accumulation. C: Dose-dependent effect of fucoidan on intracellular Aβ1–42 accumulation. D: Dose-dependent effect of leupeptin on intracellular Aβ1–42 accumulation. The level of intracellular Aβ at 30 μM leupeptin was significantly different from that of 100 μM leupeptin ($P<0.05$). Values are means ± S.D. of triplicates. *Significantly different from cells with no chloroquine treatment, $P<0.05$. ND: non-detectable.

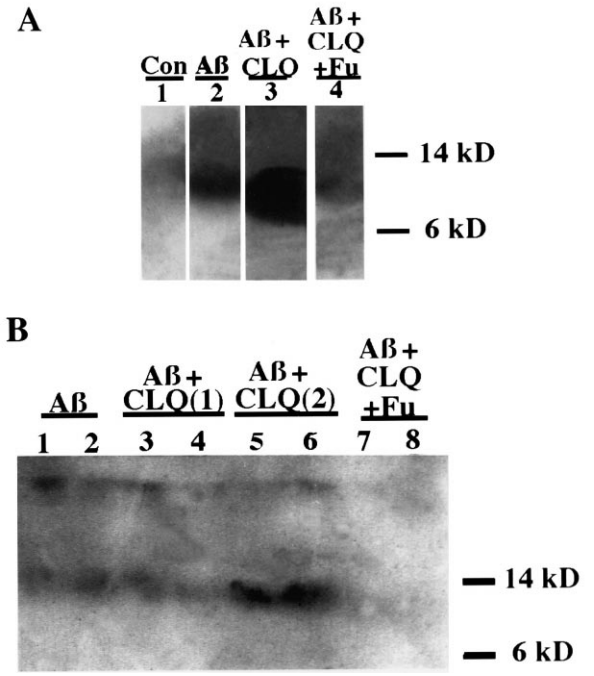

Fig. 4. A: Immunoblot of Aβ1–42 in non-trypsinized cell pellet after 24 h incubation. Lane 1: control; lane 2: 1 μg Aβ; lane 3: 1 μg Aβ+20 μM chloroquine; lane 4: 1 μg Aβ+20 μM chloroquine+400 μg/ml fucoidan. The positions of the 6 and 14 kDa molecular weight markers are shown. B: Immunoblot of Aβ1–42 in trypsinized cell pellet after 24-h in ubation. Lanes 1 and 2: 1 μg Aβ; lanes 3 and 4: 1 μg Aβ+5 μM chloroquine; lanes 5 and 6: 1 μg Aβ+20 μM chloroquine; lanes 7 and 8: 1 μg Aβ+20 μM chloroquine+400 μg/ml fucoidan.

3.3. Effect of fucoidan on intracellular Aβ1–42 accumulation

Fucoidan is a polyanionic antagonist for the type A scavenger receptors expressed by macrophages. In a previous study, 10 or 500 μg/ml of fucoidan effectively blocked the uptake of Aβ microaggregates which showed that they were mainly internalized via the scavenger receptor [12]. To test a possible role of this receptor on N9 uptake of Aβ, cells were incubated with Aβ1–42 (1 μg) and chloroquine (40 μM) together, with or without the fucoidan (50 and 400 μg/ml). After a 6 h incubation, fucoidan had no effect on the accumulation of intracellular Aβ (Fig. 3C). Therefore, the Aβ species being internalized by N9 within 6 h were mostly non-aggregated and soluble, consistent with successful initial solubilization of the applied Aβ. This suggests that in this paradigm, an alternative pathway to the scavenger receptor is being used for Aβ uptake.

3.4. Effect of leupeptin on intracellular Aβ1–42 degradation

Leupeptin is a moderate cell-permeable inhibitor for lysosomal cysteine proteases including cathepsins B, H and L [21]. When N9 microglia were incubated with Aβ1–42 (1 μg) and leupeptin (30 and 100 μM) for 6 h, intracellular Aβ1–42 levels increased substantially when compared to cells exposed to Aβ 1–42 alone (Fig. 3D; ND: non-detectable under the experimental conditions). Increasing the concentration of leupeptin to 500 μM appeared to increase Aβ1–42 accumulation compared to 100 μM of leupeptin but the difference was not statistically significant. Incubation of cells with leupeptin alone (100 μM) resulted in no detectable Aβ signal (data not shown).

3.5. Effect of chloroquine and fucoidan on accumulation of Aβ multimers (8–12 kDa)

To examine the effect of chloroquine on long-term Aβ disposition, cells were incubated with Aβ and a low concentration of chloroquine (20 μM) for 24 h. Both trypsinized and non-trypsinized cell pellets were subjected to Western blot analysis. Monomeric Aβ (4 kDa) was not detected under our Western blot conditions but multimeric Aβ (between 6 and 14 kDa) was found in both trypsinized and non-trypsinized cells. At 24 h, there is a significant amount of multimeric Aβ in Aβ-treated cells without trypsinization (Fig. 4A, lane 2) and chloroquine co-treatment markedly increased these cell-associated Aβ multimers (lane 3). A similar phenomenon was also observed in trypsinized cells (Fig. 4B) when 20 μM of chloroquine effectively augmented intracellular Aβ multimer accumulation (lanes 5 and 6), compared to Aβ alone (lanes 1 and 2) and 5 μM chloroquine (lanes 3 and 4). Interestingly, with the 24 h sample (in contrast to the 6 h sample analyzed by ELISA), fucoidan (400 μg/ml) reversed the effect of chloroquine on Aβ multimer accumulation (Fig. 4B, lanes 5 and 6 vs. 7 and 8) by decreasing the amount to a level lower than that from cells exposed to Aβ only. The Aβ multimer was not detected in either trypsinized or non-trypsinized cells upon incubation with Aβ for only 6 h, with and without chloroquine (data not shown).

4. Discussion

Characterization of AD amyloid deposits has indicated that Aβ1–42 is a major component pertinent to pathogenesis and is the initially-deposited species [22,23]. The unique behavior and physical properties of Aβ1–42 could account for its role in plaque evolution. Accordingly, Ard et al. [11] have demonstrated that exogenous Aβ1–42 was accumulated largely in the lysosomes. By using peripheral cells such as human fibroblasts [24] and rat PC12 cells [6], intracellular Aβ1–42 has been shown to remain in the lysosomes and co-localize with lysosomal markers such as lucifer yellow and horseradish peroxidase. These authors used a relatively high, aggregate-forming concentration (25 μM) of radioactive Aβ to show that Aβ1–42 was more resistant to degradation than Aβ1–40. To better mimic physiological concentrations, we have chosen lower concentrations (0.67 μM) of Aβ and found preferential accumulation of Aβ1–42 with both ELISA (Fig. 3B) and immunofluorescent labeling specific for Aβ (Figs. 1 and 2). No preferential accumulation of Aβ1–40 occurs as detected by ELISA. In this system, immunofluorescence showed that minimal Aβ1–40 may be accumulating, but it may be primarily cell surface-associated Aβ, which would be degraded by trypsin in the ELISA sample preparation. Burdick et al. [6] have suggested that Aβ1–42 accumulation could be due to its preferential adsorption (over Aβ1–40) to the cell surface and, thus, its preferential internalization. We believe that inefficient degradation of Aβ1–42 could also account for its selective accumulation because its level was significantly increased when the pH-dependent activities of the lysosomal enzymes were compromised by chloroquine [20]. The ineffective degradation and lysosomal accumulation can be attributed to the aggregation properties of Aβ1–42 [25]. Since Aβ1–42 tends to accumulate in the lysosomes, it may be more prone to lysosomal changes than Aβ1–40. Even without chloroquine, certain isolated cells accumulated abundant Aβ1–42, while others

accumulated none, suggesting that cells that begin to accumulate Aβ activate an autocrine positive feedback loop for more accumulation. Such an auto-catalytic process may involve upregulation of a receptor or Aβ1–42-induced lysosomal damage.

Microglia cells can accumulate pre-aggregated Aβ from media via the scavenger receptor [12]. Blockade of this receptor by fucoidan, however, did not alter the acute, intracellular level of Aβ1–42 in the presence of chloroquine (Fig. 3C), demonstrating an alternate pathway for Aβ uptake. Thus, the Aβ being internalized is mostly soluble and non-aggregated when present at low concentration (0.67 μM) in the medium for 6 h. The absence of Aβ multimers at 6 h further supports this hypothesis. Multimers were only detected with long exposures (24 h) to Aβ. In contrast to the pre-formed Aβ microaggregates described by Paresce [26], which are largely undegraded 72 h after uptake, the 8–12 kDa Aβ is rapidly degraded in the absence of chloroquine. Chloroquine notably increased the amount of cell-associated (Fig. 4A) and intracellular Aβ multimers (Fig. 4B). These data show that microglia cells, aside from accumulating pre-formed Aβ aggregates [11], can also accumulate soluble Aβ and may possibly provide a cellular micro-compartment for aggregate and multimer formation over time. Despite negligible effects of fucoidan on initial Aβ uptake, chronic exposure inhibits 8–12 kDa Aβ accumulation, demonstrating a probable role for scavenger receptor on multimer accumulation.

Despite the lack of effect of fucoidan on chloroquine-dependent Aβ accumulation by ELISA, fucoidan clearly attenuated the chloroquine-induced Aβ multimer accumulation. These seemingly conflicting results may relate to the facts that (1) microglia are regurgitating undegraded, intracellularly-formed aggregates (known to be enhanced with chloroquine treatment [27]) that cannot later be taken up again because of the presence of fucoidan; (2) the different time points (6 h for ELISA, 24 h for aggregates) would result in more aggregates at 24 h that could be blocked by fucoidan or (3) the polyanionic compound fucoidan can block aggregate formation by directly binding Aβ and inhibiting fibril formation.

Intracellular Aβ1–42 level was also increased upon co-incubation with leupeptin which inhibited lysosomal cysteine proteases. Our results were similar to those of Ard et al. [11] who showed increased Aβ accumulation with 100 μM leupeptin. Leupeptin is also an inhibitor for selective serine proteases. Among studies which addressed extracellular Aβ degradation by cell line conditioned medium (CM), leupeptin was shown to inhibit extracellular serine proteases [28,29]. It is thus possible for leupeptin to increase the intracellular Aβ level (Fig. 3D) by inhibiting extracellular Aβ degradation and allowing more Aβ uptake. However, this cannot account for our results because (1) chloroquine, which inhibits intracellular degradation, had a similar effect, (2) high concentrations of leupeptin were required to increase intracellular Aβ, consistent with the relatively slow leupeptin penetration into the cells, and (3) the activity of secreted proteases was low in our experimental conditions because the CM collected at 6 h had low Aβ-degrading activities (data not shown). Therefore, the contribution of extracellular serine proteases to Aβ degradation was minor.

In conclusion, disruptions of the lysosomal system enhance Aβ accumulation in microglia, consistent with data in SY5Y neuroblastoma cells [7]. Since the lysosomal degradation of Aβ1–42 is not as effective as that of Aβ1–40, disruption of lysosomal functions can retard the inherently slow Aβ1–42 degradation further which may serve as a seed for Aβ accumulation and aggregation. Therefore, SAD could possibly be potentiated by a gradual, age-related decline in lysosomal functions [30–32] which could contribute to excessive Aβ accumulation and subsequent plaque development. Cathepsin D, a lysosomal enzyme, has been reported to be a major Aβ-degrading enzyme in brain [33,34]. Its upregulation in AD and increased leakage outside the lysosome is evidence for inadequate degradation and lysosomal disruption. Detection of Aβ known to be synthesized inside cells in AD is limited by insufficient sensitivity for measuring intracellular Aβ. Therefore, failed attempts to detect intra-endosomal/lysosomal accumulations of Aβ may also relate to insufficient sensitivity, especially with Aβ1–40 and 42 antibodies. Aβ1–40 and 42 epitopes may also be rapidly removed by carboxypeptidases such as lysosomal/endosomal cathepsin B. Another explanation is that intralysosomal accumulation may induce rapid cell death followed by the removal of the Aβ-containing cell by phagocytosis, such as occurs during apoptosis. Finally, the Aβ known to accumulate outside microglial processes or on neuronal plasma membrane in Down's [35] and in early AD [36] may derive in part from Aβ exocytosed or 'regurgitated' [27] from endosomes accumulating Aβ.

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