Slow Degradation of Aggregates of the Alzheimer’s Disease Amyloid β-Protein by Microglial Cells

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Microglia are immune system cells associated with senile plaques containing β-amyloid (Aβ) in Alzheimer’s disease. Although microglia are an integral part of senile plaques, their role in the development of Alzheimer’s disease is not known. Because microglia are phagocytic cells, it has been suggested that microglia may function as plaque-attacking scavenger cells. Microglia bind and internalize microaggregates of Aβ that resemble those present in dense Alzheimer’s disease plaques. In this study, we compared the degradation by microglia of Aβ microaggregates with the degradation of two other proteins, acetylated low density lipoprotein and α2-macroglobulin. We found that the majority of the internalized Aβ in microaggregates was degraded 72 h after uptake, whereas 70–80% of internalized acetylated low density lipoprotein or α2-macroglobulin was degraded and released from cells in trichloroacetic acid-soluble form after 4 h. In the continued presence of fluorescent Aβ microaggregates for 4 days, microglia took up huge amounts of Aβ and became engorged with undegraded material. These data suggest that microglia can slowly degrade limited amounts of Aβ plaque material, but the degradation mechanisms can be overwhelmed by larger amounts of Aβ.

Alzheimer’s disease (AD) is the most frequent cause of dementia in the elderly (1). AD is a progressive, neurodegenerative disease characterized by the presence of numerous senile plaques and neurofibrillary tangles in the brain, particularly in the hippocampus and cerebral cortex (2–4). Senile plaques consist of extracellular proteinaceous deposits often associated with dystrophic neurites, astrocytes, and reactive microglia. The major component of the dense plaques is β-amyloid (Aβ), a fragment of a larger, membrane spanning glycoprotein called β-amyloid precursor protein (βAPP) (5). The predominant forms of β-amyloid are the 1–40 and 1–42 fragments.

There is substantial evidence supporting the hypothesis that progressive cerebral accumulation of Aβ is an early and perhaps necessary feature of the disease. The strongest evidence causally linking Aβ to AD is the discovery of mutations within the βAPP coding sequence that segregate with disease phenotypes in autosomal dominant familial cerebral amyloidoses. Although less than 20 families harboring these mutations have been found worldwide, these individuals have been shown to develop the classical AD-type pathology indicating that the mutations are associated with the development of AD and not a related disease (6).

All the βAPP mutations are missense changes flanking the Aβ domain and may alter the normal proteolysis that leads to production of the Aβ protein (6). Several of these AD-linked missense mutations in βAPP have been shown in cell culture (7–9) or in a transgenic mouse model (10, 11) to increase the cellular production of Aβ. Also, mutations within the presenilin-1 and presenilin-2 genes, that are responsible for the most common form of early-onset AD, appear to cause AD by influencing the proteolytic processing of βAPP (12, 13). Mutations within the two genes are associated with enhanced production of Aβ(1–42) in primary fibroblasts and increased levels of Aβ(1–42) in plasma samples obtained from patients (14, 15). Cells transfected with mutant presenilin cDNAs secrete elevated levels of Aβ(1–42) and introducing mutant presenilin-1 transgenes into transgenic mice expressing wild-type human βAPP results in overproduction of Aβ(1–42) in the brain (16).

Based on these and other observations, it is likely that aberrant metabolism of βAPP can be a primary event in the pathogenesis of AD. However, not all AD cases that result from mutations of APP are attributable to an overproduction of Aβ (17, 18). Moreover, dominantly transmitted mutations within the βAPP or presenilin genes account for only about 10% of AD cases. In most cases, AD occurs in the absence of a prior history of the disease in other family members.

One pathway of Aβ-induced neuron damage may involve inflammatory cells such as reactive microglia. Microglia found in normal adult brain are highly ramified, quiescent cells that become activated during central nervous system injury (1, 19). In the brains of patients with AD, activated microglia are associated with virtually every amyloid deposit and are concentrated in regions of compact amyloid deposits (20) where they surround and infiltrate into the β-amyloid plaques (21). Quantitative histopathology has determined that more than 80% of core plaques are associated with clusters of reactive microglia, whereas less than 2% of diffuse Aβ deposits show such an association (22). Reactive microglia are also found associated with plaques that develop in a transgenic model expressing mutant βAPP (10).

Very little is known, however, about the function of the microglia surrounding the plaques. Histological evidence has implicated microglia in AD brain as plaque-attacking scavenger cells, as sources of cytokines (23, 24), as producers of Aβ (25), and as secretors of complement proteins (26, 27). Activated microglia proliferate, exhibit phagocytic activity removing infectious agents or remnants of dying brain tissue, and
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produce a number of secreted factors such as proteases and cytotoxic agents that induce death of neurons and demyelination of oligodendrocytes (27–32). Therefore it is likely that the microglia surrounding senile plaques in AD brains are the main scavengers and removers of Aβ peptides from the brain (28).

A previous study had reported that amyloid cores isolated from the brains of AD patients are ingested by cultured microglia, and then collected and stored in phagosomes. Some of the ingested, non-degraded amyloid remained within the phagosomes for up to 20 days, suggesting a very limited effectiveness of microglia in degrading Aβ fibrils (25). Amyloid fibrils were observed in microglial phagosomes as demonstrated by light microscopy, immunocytochemistry, and electron microscopy. However, degradation of the fibrils was not measured directly or quantified. It has also been reported that synthetic Aβ peptides accumulate in cultured human skin fibroblasts and are resistant to degradation for 3 days (33). A different result, however, was obtained in a study that used microglia to degrade Aβ peptides. It was reported that rat microglial cultures and an activated human monocyte cell line could degrade Aβ(1–42) to the culture medium. The ability of cells to degrade soluble Aβ was evaluated by adding synthetic Aβ(1–42) to cell cultures and then measuring the amount of Aβ remaining in the medium after an 8-h incubation with the cells. Degradation of Aβ was measured by the loss of immunoreactive material in Western blots. It was also found that microglia could remove Aβ fibrils immobilized as plaque-like deposits on tissue cultures (18). The amount of immobilized Aβ was assessed by phase-contrast imaging and thioflavin T staining of the dots before and after incubation with the cells. However, the question of whether the decrease in Aβ was due to degradation or just to internalization by the cells was not addressed directly.

We had found that microglia from mouse central nervous system rapidly internalize aggregates of Aβ(1–42) peptides that, by electron microscopy, resemble the amyloid fibrils found in AD plaques (34). Using an in vitro model in which Aβ microaggregates were added to cell culture media, we found that primary cultures of microglia internalize aggregates of fluorescently labeled or radioiodinated Aβ peptide. This uptake was mediated by a type A scavenger receptor that also mediated internalization of acetylated low density lipoprotein (Ac-LDL). Binding of Aβ to scavenger receptors was also reported by El-Khoury et al. (35).

In this paper we examine the degradation of those Aβ fibrils taken up by microglia via the scavenger receptor. We carried out kinetic comparisons of the degradation rate of Aβ aggregates to that of two other proteins previously shown to be internalized by microglial cells via receptor-mediated endocytosis (34). We found a slow but significant degradation of Aβ microaggregates by microglia. The balance between degradation and production may be important in the net accumulation of plaque proteins in AD.

EXPERIMENTAL PROCEDURES

Isolation of Microglia—We prepared primary cultures of mixed glia from newborn mice and then isolated the weakly adherent microglia from cell monolayers according to previously described methods (28, 36). To prepare primary cultures of mixed glia we obtained neocortical tissues of newborn mice, removed the meninges, minced and incubated the tissues in 2.5% trypsin (Worthington Biochemical Corp., Freehold, NJ) and 0.01% deoxyribonuclease 1 (DNase) (Worthington) in phosphate-buffered saline for 5 min at 37 °C. The tissue was triturated with fire-polished pipettes in 0.1 μg/ml DNase in Dulbecco’s modified Eagle’s medium (DMEM) (Life Technologies, Inc.) with 10% fetal bovine serum (Life Technologies, Inc.). The cells were centrifuged for 5 min at 350 × g, and the pellet was resuspended in DMEM with 10% fetal bovine serum and penicillin/streptomycin. The supernatant was passed through a 145-μm mesh, re-centrifuged for 5 min, resuspended, passed through a 33-μm mesh, and centrifuged one more time. Cells were resuspended in growth medium then plated in 75-cm² flasks coated with poly-d-lysine at a density of about 1.5–2.0 × 10⁶ cells per flask (about 5 cortices per flask). The mixed glial cultures were grown in bicarbonate-buffered DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.) at 37 °C in a 5% CO₂ humidified air atmosphere. The mixed glial cultures were grown for 1 week before microglia were collected from the flasks. Microglia were harvested by orbital shaking for 20 min in DMEM or where noted, in DMEM with 12 mM lidocaine (Sigma). Cells were centrifuged for 10 min at 350 × g and plated onto 35-mm diameter plastic tissue culture dishes in which a 7-mm diameter hole was punched in the bottom and a polysyline-coated number 1 coverslip was attached beneath the hole (37). Cells were grown at a density of 10⁴–10⁵ cells/coverslip. Shaking one 75-cm² flask of mixed glial cultures usually yielded enough microglia for 10 coverslips. For 125I-Aβ uptake experiments, microglia were plated on poly-d-lysine coated 24-well trays at the same density (10⁶ cells/well).

We used the uptake of fluorescent acetylated low-density lipoprotein (DiI-Ac-LDL) by microglia to assess the purity of our microglial cultures because microglia, unlike astroglia or oligodendroglia, have the Ac-LDL receptor (28). We found that over 95% of the cells harvested after shaking took up DiI-Ac-LDL. Astroglia were identified by immunostaining for glial fibrillary acidic protein. Anti-glia fibrillary acidic protein antibodies were provided by Dr. Wilma Friedman (Columbia University). We found that our enriched microglial cultures contained less than 1% of glial fibrillary acidic protein positive astroglia.

We used the methods described by Giulian et al. (22) to distinguish microglia from blood macrophages and found no contamination by other types of macrophages. Just after seeding microglia were round, but after a day in culture, the cells exhibited two predominant morphologies: an elongated, ramified form and an ameboid form. Ramified cells are generally viewed as resting or quiescent microglia that lack phagocytic properties (28), but we found the two cell types did not differ in their ability to bind and take up DiI-Ac-LDL.

Proteins—1–42 Aβ-amylloid peptide was purchased from Bachem (Torrance, CA). The lyophilized powder was diluted in sterile water to 5 μg/ml and kept at −80 °C. Fluorescent β-amylloid was prepared by derivatizing it with Cy3, an orange fluorescing carboxyamine dye (Biochemical Detection Systems Inc., Pittsburgh, PA) according to the manufacturer’s instructions. Aβ(1–42) was dissolved at 1 mg/ml in 0.1 M sodium carbonate/sodium bicarbonate buffer, pH 9.3, then added to the Cy3 Acetyl-LDL. The labeled protein was then added to the cells and incubated with Cy3 dye. The labeled protein was separated from excess, unconjugated dye by dialysis. This stock of Cy3-Aβ at pH 9.3 was kept at 4 °C.

125I-Labeled Aβ was prepared using the chloramine-T method as described (34, 38). 500 μg of Aβ was iodinated in 0.05 M sodium borate buffer, pH 9.0. The excess 125I was removed by passage over a G-15 Sephadex column and dialysis against borate buffer for 6 h at room temperature. The specific activity of the 125I-labeled Aβ was 713 cpm/μg.

Ac-LDL and αM were iodinated using the same chloramine-T method (38). The specific activity of 125I-labeled Ac-LDL was 796 cpm/ng and that of αM was 276 cpm/ng. It has been shown that these iodinated proteins retain the ability to bind specifically to their receptors (38, 39).

Acetyl-LDL was prepared by acetylation of LDL with acetic anhydride as described previously (40) and provided by Dr. Ira Tabas (Columbia University, NY). Acetyl-LDL was labeled with 1,1‘-dioctadecyl-3,3,3’-tetramethylindocarbocyanine perchlorate (DiI) ( Molecular Probes, Eugene, OR) as described (41). αM-Macroglobulin (αM) was purified, converted to the receptor-binding form, and conjugated to either fluorescein isothiocyanate (FITC) or Cy3 as described previously (42) or Cy3 (Biological Detection Systems Inc.) according to the manufacturer’s instructions. FITC-labeled, fixable, 70-kDa dextrans were purchased from Molecular Probes (Eugene, OR).

For all our studies on the uptake of Cy3-labeled Aβ and unlabeled Aβ microaggregates, the peptide was pre-aggregated before being added to microglial cultures. Aβ was initially diluted in water while vortexing, mixed well, then further diluted in labeling medium (serum-free DMEM medium without supplements, buffered to pH 7.4, and containing 100 units/ml penicillin and 100 μg/ml streptomycin). Labeling medium also contained 10 mg/ml bovine serum albumin radiimmunoassay grade (Sigma). Aβ peptide was allowed to aggregate for 1 h at room temperature, and Cy3-Aβ was allowed to aggregate at room temperature for 15 min before being added to microglial cultures.
Fluorescent Labeling of Cells—All labeling was done in air at 37 °C. Prior to any fluorescent labeling, the cells were rinsed twice with dye-free labeling medium. We diluted the Cy3-Ab in pH 7.4 labeling medium (DMEM with 10 mg/ml bovine serum albumin), allowed it to aggregate for 15 min at room temperature, and then added the medium to the microglia in coverslip dishes. The cells were incubated in the same way with Cy3-labeled αM in labeling medium. After the cells were incubated for a short time (5–15 min) with labeling medium containing fluorescently-labeled peptides, the medium and fluorescent peptides were washed away. The cells were then incubated in medium with no added peptides, rinsed 3 times in labeling medium, 3 times with chase medium with no serum, then 3 more times with serum containing chase medium which was microglial culture medium. The cells were fixed with 5% paraformaldehyde freshly diluted in medium 1 (150 mM NaCl, 20 mM HEPES, 1 mM CaCl₂, 5 mM KCl, 1 mM MgCl₂, pH 7.4).

Uptake of 125I-Labeled Ligands—Cells were grown in 24-well plates for 2–3 days before the start of the experiment. 125I-Labeled Ab (1 μg/ml) was formed into aggregates as described for Cy3-Ab. The cells were washed three times with labeling medium and then incubated with the radiolabeled peptides for 1 h at 37 °C in a 5% CO₂ humidified air atmosphere. The cells were rinsed extensively as described for the fluorescent labeling experiments, then incubated with chase medium for varying times. At the end of the chase, the medium bathing the cells was removed and the radioactivity was measured. The cells were rinsed three times in chase medium then three times with phosphate-buffered saline and solubilized with 1 M NaOH. The chase medium was precipitated three times in chase medium then three times with phosphate-buffered saline and solubilized with 1 M NaOH. The chase medium was precipitated with 10% trichloroacetic acid on ice for 1 h and centrifuged to separate the soluble fraction from the trichloroacetic acid precipitable fraction. The radioactivity of each fraction was counted. Radioactivity was measured in a γ-counter, and the protein content of each well was measured using the Pierce BCA protein assay reagent. Where indicated, excess Ac-LDL or excess αM were added as competitive inhibitors to the incubation medium along with the radiolabeled peptides and maintained in the labeling medium for the entire incubation. The radioactivity of these background dishes was subtracted from the values for the control experiments. All radioactive experiments were conducted with 3 wells per condition and repeated at least three times on separate days.

Immunofluorescence—Microglia were rinsed with labeling medium, then incubated with unlabeled Ab microaggregates (5 μg/ml) or unlabeled αM (50 μg/ml) for 1 h at 37 °C. The cells were rinsed three times with labeling medium, fixed, permeabilized with 0.05% Triton X-100, then incubated for 1 h at room temperature with a monoclonal antibody specific to Ab (Senetek PLC, St. Louis, MO) or αM (Genzyme Diagnostics, Biotech Subsidiary, San Carlos, CA). The cells were rinsed three times over 15 min, then incubated for 1 h at room temperature with a FITC-conjugated goat anti-mouse IgG (Pierce, Rockford, IL). Control dishes were incubated with only the secondary antibody. Antibodies used for Ab immunofluorescence were diluted in phosphate-buffered saline with 1% bovine serum albumin and 3% normal goat serum. Antibodies used for αM immunofluorescence were diluted similarly but instead of serum, with 1% goat anti-rabbit IgG.

Quantitative Fluorescence Microscopy—Fluorescence microscopy and digital image collection were performed using a Leitz Diavert fluorescence microscope equipped with a Photometrics (Tucson, AZ) cooled CCD camera as described previously (43). Fluorescence quantification was carried out with a × 25 magnification objective to obtain a large number of cells per field, whereas the images for visualization purposes were obtained at a higher magnification (× 63, NA 1.4 objective) as described previously (44). Fields used for quantification were selected at random throughout the dish and focused using phase-contrast optics before viewing the fluorescence. Digital fluorescence images were obtained, and the background signal was removed from the images as described previously (34, 45). Cells were identified in the image plus the standard deviation of the pixel intensity. Objects of the size of cells and above the threshold intensity were identified using Metamorph software routines (Universal Imaging, West Chester, PA). Total fluorescence intensity was taken as the sum of all intensity in these objects, which then was divided by the number of cells in the field to calculate the average fluorescence intensity per cell. The percentage of total intensity per cell remaining after various chase times was determined for Ab microaggregates and αM.

Confocal Microscopy—Fluorescence images of cells were obtained with a Bio-Rad MRC 600 laser scanning confocal microscope (Bio-Rad Microscience, Cambridge, MA) as described previously (46).
fluorescence did not change), but after 30-min incubations in peptide-free medium the total cell fluorescence began to decrease (Fig. 1C). After a 6-h chase, we found almost no internal fluorescence (Fig. 1E). The decrease in cell internal fluorescence was not due to cell death because we found no change in cell morphology or number during the course of the experiments as shown in the phase-contrast images (Fig. 1, B, D, and F).

Microglia pulsed with Cy3-labeled Aβ Microaggregates Retained Internal Cy3 Labeling for Several Days—We performed similar pulse-chase experiments with Cy3-labeled Aβ microaggregates. Because the Aβ-(1–42), which are minor components of the Aβ in human cerebrospinal fluid and plasma, are critically important in AD where they deposit selectively in all types of senile plaques (47) in which most of the Aβ ends at Aβ-(42) (48, 49), and since Aβ-(1–42) forms fibrils more rapidly than Aβ-(1–40) (50), we used synthetic Aβ-(1–42) in our experiments to model AD amyloid plaques in vitro. Cy3-Aβ was incubated to form fibrillar aggregates in pH 7.4 labeling medium as described under “Experimental Procedures.” Synthetic peptides corresponding to Aβ have been shown by others to spontaneously form insoluble, amyloid-like fibrils (50–53). We have shown previously by electron microscopy that, the fibrillar morphology of the Aβ microaggregates formed under the conditions described under “Experimental Procedures” resembles the morphology of the amyloid filaments observed in senile plaques (34).

Adherent microglia were incubated for 5–15 min with 1 μg/ml fluorescently labeled Aβ microaggregates. The labeling medium containing Cy3-labeled Aβ microaggregates was then rinsed off, and growth medium containing serum was added back to the cell cultures. After the short pulse with Aβ microaggregates, microglia were kept in growth medium at 37 °C for periods of up to 1 week. Immediately after the pulse with Cy3-labeled Aβ microaggregates, microglia were brightly labeled with Cy3 fluorescence in a pattern identical to that seen with Cy3-labeled α2M (34). The fluorescence was initially concentrated in numerous discrete vesicles in the cell periphery (Fig. 2A), and with longer chase times it moved into perinuclear vesicles (Fig. 2, C–F). After the cells were rinsed and then chased in growth medium, intracellular fluorescence became increasingly concentrated in larger perinuclear vesicles. The change in intracellular fluorescence over time was observed using two methods. Live cells were observed by fluorescence microscopy every day during the course of the experiment, and every day a sample of cells was fixed and observed by fluorescence microscopy. At first (chase times of up to 1 h), the Cy3-labeled Aβ microaggregate staining pattern was identical to that seen with Cy3-labeled α2M. However, within 4 h of chase it was apparent that these two proteins had very different cellular fates after internalization. We observed little, if any, decrease in internal fluorescence in microglia pulsed with Cy3-labeled Aβ microaggregates even after 1 week of incubation in medium containing no labeled peptides (Fig. 2, C–F). For 1 week, the cells continued to show very bright internal fluorescence concentrated in large perinuclear vesicles, morphologically similar to lysosomes.

Internalized Cy3-labeled Aβ Microaggregates Co-localize with Pre-internalized FITC-dextran after a 1-h Chase—We used confocal microscopy to observe the intracellular location of the internalized Cy3-labeled Aβ microaggregates. We pulsed microglia with 1 mg/ml fixable 70-kDa FITC-dextran for 45 min and chased for 1.5 h to allow the dextrans to reach the late endosomes and lysosomes (54, 55). Cy3-labeled Aβ microaggregates (10 μg/ml) were then added to the medium, and the cells were incubated with Aβ microaggregates for 1 h. Cells were then chased in label-free medium for varying times, fixed, and viewed by confocal microscopy. Fig. 3 shows a single focal plane of the microglia incubated with FITC-dextran and Cy3-labeled Aβ microaggregates. We found that after a 1-h chase, most of the vesicles containing Cy3-Aβ (Fig. 3B) also contained pre-internalized FITC-dextran (Fig. 3A). This shows that within 1 h, much of the internalized Cy3-labeled Aβ microaggregates has reached the acid hydrolase containing late endosomes and lysosomal compartments. When microglia were chased for 2 days, the majority of the Cy3-labeled Aβ microaggregates (Fig. 3D) were still found in FITC-dextran-labeled lysosomes (Fig. 3C).

Internalized 125I-labeled Aβ Microaggregates Were Degraded at a Much Slower Rate Than Either 125I-labeled α2 or 125I-labeled Ac-LDL—To quantify our observations on the stability of intracellular Aβ microaggregates, we performed pulse-chase experiments with 125I-labeled Aβ microaggregates and measured the release of the 125I label from the cells into the medium. We compared the rate of release of 125I from cells incubated with 125I-labeled Aβ microaggregates to that of cells incubated with 125I-α2M or 125I-Ac-LDL. For every time point, we incubated two sets of cells with radiolabeled Aβ microaggregates. One set contained only 125I-labeled Aβ microaggregates, the other contained the labeled proteins as well as excess unlabeled Ac-LDL. From our previous studies (34) using Ac-LDL, we know that excess Ac-LDL blocks uptake of 125I-labeled Aβ microaggregates. This background of cell surface 125I-labeled Aβ was subtracted from the total counts. The binding of 125I-labeled Aβ microaggregates in the presence of 100 μg/ml Ac-LDL was less than 10% of control binding. We also used excess Ac-LDL to block 125I-labeled Ac-LDL uptake and excess activated α2M to block 125I-labeled α2M uptake, and these values were subtracted from the total counts at each
time point.

Microglia were incubated for 1 h with $^{125}$I-labeled Aβ microaggregates, $^{125}$I-labeled $\alpha_2$M or $^{125}$I-Ac-LDL. After incubation with labeled proteins, cells were washed and chased for varying times. The radioactivity in the chase medium and the cells was measured. 72 h after uptake, 70–80% of the $^{125}$I-labeled Aβ microaggregates were still cell associated (Fig. 4A). In comparison, within 4 h 80–90% of the total radioactivity from internalized $^{125}$I-labeled Ac-LDL and $\alpha_2$M was released into the medium (Fig. 4A).

The loss of radioiodinated proteins from cells could be due to either proteolytic degradation or release of intact proteins. To distinguish these possibilities, the degradation of $^{125}$I-labeled proteins was measured by the release of 10% trichloroacetic acid-soluble iodotyrosine into the medium. There was a rapid release of trichloroacetic acid-soluble material from cells incubated with $^{125}$I-labeled Aβ microaggregates but did not detach microglial cell microaggregates (Fig. 4B). The continued uptake of extracellular Aβ microaggregates was significantly slower than that of $^{125}$I-labeled $\alpha_2$M or $^{125}$I-labeled Ac-LDL (after 4 h over 70% of the total radioactivity was in the chase medium and was trichloroacetic acid soluble) (Fig. 4B).

Although the rate of degradation of $^{125}$I-labeled Aβ microaggregates was significantly lower than that of $^{125}$I-labeled $\alpha_2$M or $^{125}$I-labeled Ac-LDL, a fraction of $^{125}$I-labeled Aβ microaggregates was degraded, releasing trichloroacetic acid-soluble $^{125}$I-tyrosine (Fig. 4B). This slow degradation persisted throughout the 72-h chase. Chloroform extraction of the trichloroacetic acid-soluble chase medium showed that over 95% of the radioactivity remained in the aqueous layer, indicating that there was not a significant amount of free $^{125}$Iodine in the chase medium (data not shown).

Cells did release some nondegraded $^{125}$I-labeled peptides into the medium which could be precipitated by trichloroacetic acid (Fig. 4C). Release of internalized $\alpha_2$M by fibroblasts has been reported previously (56–58) and was similar in magnitude to the release from microglia.

The continued uptake of extracellular Aβ microaggregates stuck to the dish or cell surface (i.e. Aβ microaggregates that were not washed away by rinsing before the chase) was not a significant factor in the degradation kinetics. We pulsed microglia with radioiodinated Aβ microaggregates, trypsined the cells to remove external Aβ microaggregates, and then chased in growth medium. It had been previously reported that cell surface-adsorbed Aβ peptide is effectively removed (>95%) by trypsin digestion (33), and we verified this using Cy3-labeled Aβ microaggregates. Trypsin treatment removed all extracellular Aβ microaggregates but did not detach microglial cell cultures from the dishes. We found that trypsin treatment had no significant effect on our results.

The increase of iodotyrosine in the culture medium could be due to intracellular catabolism or it could be due to degradation by secreted proteases. It has been reported that a serine protease $\alpha_2$M complex found in culture medium could degrade soluble Aβ (11). We examined whether there were any such
proteases in our chase medium that could degrade the three \(^{125}\)I-labeled proteins. We adsorbed \(^{125}\)I-labeled peptides to coverslip dishes with no cells, then added chase medium or conditioned medium from 1 week of microglial culture to the dishes. These dishes were incubated in parallel with dishes containing microglia, and at each of the time points the amount of degradation of the attached proteins was measured. Adsorbed proteins were removed by 1 M NaOH, and both the medium and the attached peptides were trichloroacetic acid precipitated. We found no significant decrease in total radioactivity over time and no release of trichloroacetic acid-soluble material (data not shown), indicating that degradation of radiolabeled peptides was intracellular and not due to proteases in the medium.

**Cells Degrade Unlabeled Aβ Microaggregates at a Much Slower Rate Than Unlabeled \(\alpha_2\)M**—To verify that the degradation properties of Aβ and \(\alpha_2\)M are unaltered by their fluorescent or radioactive tags, we used unlabeled proteins in similar pulse-chase experiments. Microglia were pulsed for 1 h with unlabeled Aβ microaggregates or \(\alpha_2\)M. After 0, 3, 6, and 24 h chase times the cells were fixed, permeabilized, and analyzed by immunofluorescence. The immunofluorescence localization of internalized \(\alpha_2\)M and microaggregates of Aβ are shown in Fig. 5 (A and B). After a 6-h chase, almost no \(\alpha_2\)M could be detected in the cells by immunofluorescence (Fig. 5C). In contrast, microaggregates of Aβ could be seen by immunofluorescence even after a 24-h chase (Fig. 5D). We carried out a quantitative analysis of the immunofluorescence experiments as described under “Experimental Procedures.” Intensity measurements were made on 8–10 fields of cells containing approximately 25 cells/field for each condition from three different experiments. The fluorescence intensity of \(\alpha_2\)M fell to the background level after 6 h. However, after 24 h chase, the average immunofluorescent intensity of Aβ microaggregates in three experiments was at 73 ± 14% (S.E.) of the initial value. This is in good agreement with the degradation data obtained with \(^{125}\)I-labeled Aβ microaggregates. These data indicate that the persistence of Aβ microaggregates in cells after internalization is not a consequence of the labeling of the peptides.

**Microglia Become Engorged with Aβ in Long-term Incubations with Aβ Microaggregates**—We incubated microglia in the continuous presence of fluorescently labeled Aβ microaggregates or fluorescently labeled \(\alpha_2\)M for periods of up to 1 week. The cells were observed every day as described for the pulse-chase experiments. We found that within 4 h the Cy3-labeled \(\alpha_2\)M reached a steady state in which uptake was balanced by degradation and efflux. Internal Cy3-\(\alpha_2\)M labeling did not change greatly over time. In fact, for 2 weeks, the cells continued to take up Cy3-labeled \(\alpha_2\)M, and the staining pattern remained similar to the pattern seen after a 15-min incubation (compare Fig. 6, B with D). Microglia incubated in the continuous presence of Cy3-labeled Aβ microaggregates also continued to take up Aβ microaggregates, but the effect on the cells was very different. The cells accumulated Cy3 label and became progressively brighter and larger (Fig. 6C). With Aβ, the degradation was slow, so continued uptake caused massive accumulation. After 4 days with Aβ microaggregates, microglia became enlarged and filled with labeled Aβ (Fig. 6C). Microglia incubated for 4 days with Cy3-labeled Aβ microaggregates became 20–50 times brighter than microglia incubated for only 15 min with Aβ microaggregates. To visualize the cells, the intensity in panel C was decreased to 2% of its actual value.

**DISCUSSION**

Using fluorescence microscopy we found that aggregated Aβ(1–42) was rapidly internalized by primary cultures of murine microglia. We had found previously that microglia internalized Aβ fibrils by a type A macrophage scavenger receptor (34). In AD, there is a strong expression of the scavenger receptor on activated microglia in the vicinity of senile plaques (59), and this suggests that microglia are engaged in endocytosis or phagocytosis of Aβ containing senile plaques.

Here we report that Aβ microaggregates internalized by the scavenger receptor accumulated intracellularly in microglia. This accumulation of Aβ microaggregates was due not only to the high rate of uptake via the scavenger receptor, but also to the subsequent slow rate of degradation of the microaggregates by microglia. When we examined the rate of degradation of internalized Aβ microaggregates, we found that although mu-

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**Fig. 5.** Unlabeled Aβ microaggregates degrade at a much slower rate compared with unlabeled \(\alpha_2\)M. Cells were incubated for 1 h with 5 \(\mu\)g/ml unlabeled Aβ microaggregates (B and D) or 50 \(\mu\)g/ml unlabeled \(\alpha_2\)M (A and C). The cells were then rinsed and fixed immediately (A and B) or incubated in growth medium for varying chase times and then fixed. After fixation, the cells were permeabilized and subjected to immunofluorescence using monoclonal antibodies against \(\alpha_2\)M (A and C) or Aβ (B and D). Images shown are digital fluorescence micrographs that have been corrected for background as described under “Experimental Procedures.” Representative fields for 6 h (C) and 24 h (D) chase times are shown. Bar, 10 \(\mu\)m.

**Fig. 6.** Microglia become engorged with Aβ in long-term incubations with Aβ microaggregates. Microglia were incubated with 20 \(\mu\)g/ml Cy3-labeled Aβ microaggregates (A and C) or Cy3-labeled \(\alpha_2\)M (B and D) for 15 min (A and B) or 4 days (C and D). Digital fluorescence micrographs of the cells are shown. To present the data, the intensity in panel C was decreased to 2% of its actual value. Microglia incubated with Cy3-labeled Aβ microaggregates continued to accumulate internal Cy3 label over time while cells incubated with Cy3-labeled \(\alpha_2\)M showed no increase in internal fluorescence. Bar, 10 \(\mu\)m.
rine microglia could rapidly internalize microaggregates of Aβ peptide and deliver them to degradative organelles, degradation of the Aβ microaggregates was slow. When microglia were pulsed briefly with fluorescent Aβ microaggregates, then chased in Aβ-free medium, Aβ fluorescence was increasingly concentrated in lysosomes with time. However, even after a 1-week chase, a large proportion of Aβ fluorescence persisted in the microglial lysosomes, whereas fluorescently labeled α-M was degraded within 6 h. Similarly, we found that internalized 125I-labeled Aβ microaggregates were degraded at a much slower rate than either 125I-labeled α-M or 125I-labeled Ac-LDL. The slow degradation was not due to covalent labeling of the peptides since unlabeled Aβ microaggregates also showed slow degradation when detected by immunofluorescence.

There is substantial evidence supporting the hypothesis that Aβ deposition plays a role in initiating AD lesions that ultimately lead to neuronal dysfunction and then dementia (11). In AD, neuronal degeneration is preceded by the accumulation of amyloid plaques in the cerebral and limbic cortices, and it has been shown that the number of senile plaques correlates with the severity of the disease (60). As a result, many studies have focused on finding compounds that decrease Aβ production, prevent its aggregation into potentially cytotoxic amyloid fibrils, protect cells from Aβ-mediated neurotoxicity, or enhance Aβ clearance from the brain. Most of these studies have focused almost entirely on the biosynthetic processing of AβPP and the production and fibrillogenesis of Aβ even though the degradation of the peptide is probably of equal importance in the process leading to progressive amyloid deposition (11).

Aβ isolated from senile plaques is hydrophobic, has limited solubility even in strongly denaturing aqueous solvents, and is resistant to crude collagenase (61) and pepsin (62) digestion. The increase in Aβ plaques in AD brains could be due to several factors: 1) an increase in cellular production of Aβ peptides; 2) an increase in the formation or stability of Aβ aggregates or fibrils; or 3) a decrease in the normal clearance of Aβ peptides or aggregates. The presence of activated microglia within amyloid plaque cores has led to proposals that microglia may contribute either to the formation of the plaques (63) or to the clearance and phagocytosis of the amyloid fibrils (59). There have been several immunochemical analyses characterizing the relationship between plaque microglia and amyloid fibrils (25, 63–65), but the mechanisms of degradation of Aβ aggregates remain uncertain.

A previous study had found that rat microglia, astrocytes, and a human THP-1 monocyte cell line could degrade Aβ(1–42) added to the culture medium (18). A different result, however, was obtained in a study that used cultured microglia to degrade senile plaques. It was found that senile plaques isolated from brains of AD patients are ingested by cultured microglia and then collected and stored in phagosomes. Some of the ingested, non-degraded senile plaques remained within the phagosomes for up to 20 days (25, 63). We find that Aβ(1–42) fibrils are in fact degraded but very slowly. Thus, the Aβ(1–42) fibrils which are the main component of AD senile plaques, are by themselves not easily digested by microglia, even in the absence of other senile plaque proteins or other factors that may affect the aggregation, uptake, and degradation of the fibrils.

The rapid internalization of Aβ microaggregates, coupled with the slow degradation can lead to net accumulation of Aβ in cells. We found that incubating microglial cell cultures with low amounts of Aβ microaggregates (5–20 μg/ml) over a period of several days, could result in huge accumulation of the Aβ microaggregates inside the cells. The microglia in culture were taking up small (less than 400 nm) (34) fibrils and concentrating them in acidic late endosomes and lysosomes. After a few days some of these intracellular deposits become quite large (up to 2 μm, Fig. 6C). Thus, under conditions of net accumulation; microglia may be playing a role in the formation of large, dense Aβ deposits. Small changes in the amount of Aβ microaggregates being taken up or in the rate of degradation could have a large effect on the net accumulation of Aβ over long periods of time. The engorgement of microglia with undigested Aβ is strikingly similar to the conversion of macrophages into foam cells when excessive amounts of cholesterol are taken up and stored as cholesterol ester droplets (66–73). As with the Aβ-loaded microglia, these cholesterol droplets can occupy a large fraction of the cytoplasmic volume. It is noteworthy that conversion to foam cells leads to release of proinflammatory mediators (74–77), a process that has also been proposed to occur in microglia exposed to AD plaques (27, 31, 32, 78).

Thus, activated microglia within amyloid plaque cores may be involved not only in clearance of Aβ but may contribute to the pathology of AD. For example, microglia may directly contribute to amyloidosis by participating in the formation and growth of Aβ plaques. Alternatively, Aβ may activate microglia and trigger the production of proinflammatory and potentially cytotoxic mediators. Both of these possibilities are yet to be further investigated.

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