Rapid cellular uptake of Alzheimer amyloid βA4 peptide by cultured human neuroblastoma cells

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Abstract Cerebral deposition of βA4 (β-amyloid) peptide is a major pathological feature of Alzheimer's disease. Although the mechanism of βA4 production from cells has been investigated extensively, so far little is known about the catabolism of the peptide. We report here that the human neuroblastoma cell line SH-SY5Y can rapidly clear βA4 in the culture medium. The clearance was not due to the degradation by extracellularly released protease, but rather due to intracellular degradation after cellular uptake. This clearance activity was specific to SH-SY5Y cells among several cell types examined. Some of the βA4-derived peptides lacking the N-terminal part of the molecule were not catabolized, suggesting a specific interaction between the cells and βA4. Although most of the peptide was degraded after uptake, small amounts of peptide was accumulated in insoluble fractions of the cells and remained stable for several days. These observations suggest that this uptake-degradation activity may contribute to AD pathogenesis in two different ways: either to prevent the amyloid deposition by reducing extracellular βA4 concentrations, or to promote the deposition by producing insoluble seeds for amyloid formation.

Key words: Alzheimer's disease; βA4 amyloid; Cellular uptake; Neuroblastoma cell

1. Introduction

Amyloid deposits in the brain are the central neuropathological features of Alzheimer's disease (AD). βA4 (β-amyloid) peptide was identified as the major component of these insoluble aggregated structures called senile plaques [1,2]. This hydrophobic peptide with 40-42 amino acid residues is produced by proteolytic processing of a larger transmembrane protein termed amyloid precursor protein (APP) [3]. Point mutations in the APP gene and recently identified presenilin genes, which are associated with familial AD patients, have been found to cause increased production of βA4, especially its 'long-tailed' variant βA4 1-42 [4–7]. It is also reported that transgenic mice expressing the mutant APP protein develop the characteristic amyloid pathology of AD [8]. Together with the fact that βA4 deposition is the earliest structural sign of AD pathology, which may precede the disease symptoms by several decades, it is widely accepted that this molecule plays a pathogenic role in AD.

βA4 was found to be a product of normal cell metabolism and is also detected as a soluble molecule in body fluids of both AD patients and healthy controls [9–11]. However, the mechanism regulating the in vivo level of βA4 is not elucidated. Especially, compared to the cellular synthesis of βA4, very little is known about its catabolism, which can be another critical factor of plaque formation. Here we show that the human neuroblastoma cell line SH-SY5Y cells can rapidly degrade βA4 peptide through a cellular uptake mechanism.

2. Materials and methods

2.1. Cell culture

SH-SY5Y cells [12] were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum (FCS), nonessential amino acids (Sigma, USA), penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were cultured in 25 cm^2 flasks and routinely passaged by using trypsin-EDTA solution. N2a cells, COS7 cells, and HeLa cells were also maintained in the same medium. Cells were subcultured into individual wells of 96-well plates (for medium βA4 analysis) or 24-well plates (for cell-associated βA4 analysis) 1–2 days before the experiments and used at 70–80% confluence (approx. 2×10^4 cells/well for 96-well plate and 1×10^5 cells/well for 24-well plate).

2.2. Analysis of βA4 clearance from cell culture medium

Medium of cells cultured in 96-well plate was replaced with 100 μl of fresh DMEM medium (supplemented with 1% FCS) containing each βA4 peptide. Cells were incubated at 37°C in 6% CO_2, and at each time point, 5 μl of the medium was withdrawn and analyzed by Western blotting.

2.3. Analysis of cell-associated βA4

Medium of cells cultured in 24-well plates were replaced with fresh DMEM (supplemented with 1% FCS) containing 50 ng/ml βA4 1-40 and incubated for 24 h. Cells were washed with cold PBS (2 ml×3 times) and lysed by the addition of 300 μl of ice-cold cell lysis buffer (50 mM Tris-HCl pH 7.4 containing 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 2 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mg/ml leupeptin and 0.5 mg/ml pepstatin). Samples were transferred to fresh eppendorf tubes, incubated on ice for 15 min and centrifuged at 13000 rpm at 4°C for 10 min. βA4 in the supernatant (cell lysate) was first immunoprecipitated with anti-βA4 monoclonal antibody (clone W0-2 [13]) and protein G-agarose (Boehringer Mannheim, Germany), and analyzed by Western blotting. βA4 in the precipitate (cell pellet) was solubilized by the addition of 3×sample loading buffer (6% SDS, 15% 2-mercaptoethanol, 30% glycerol and 0.3 mg/ml bromophenol blue in 188 mM Tris-HCl, pH 6.8) and analyzed by Western blotting.

2.4. Analysis of the stability of internalized βA4

SH-SY5Y cells in 24-well plates were incubated with 50 μg/ml of βA4 1-42 in DMEM (supplemented with 1% FCS) for 24 h. Cells were washed with fresh DMEM medium (without βA4) (2 ml×3 times) and cultured further for 1–6 days in DMEM (1% FCS) without βA4 addition. At each time point, cells in one well were washed with cold PBS, lysed and analyzed as described above.

2.5. Western blotting

Western blot detection and quantitation of βA4 was performed as described elsewhere [13]. Briefly, heat-denatured samples were separated by 10–20% or 16% Tris-Tricine SDS-PAGE. Proteins were

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blotted onto nitrocellurose membrane and detected with sequential incubation with monoclonal anti-βA4 antibodies, HRP-labeled anti-mouse Ig secondary antibody and ECL detection solution (Amersham, UK). βA4 concentrations were quantitated by densitometric analysis of the bands using MacBas program (Fuji, Japan). The following monoclonal antibodies were used for the detection [13]; clone W0-2 (anti-βA4 1-16) for the detection of βA4 1-40, 1-42 and 1-28; clone G2-10 (anti-βA4 C-terminus 40) for the detection of βA4 6-40 and 17-40 and clone G2-11 (anti-βA4 C-terminus 42) for the detection of 17-42 and internalized βA4 1-42.

3. Results

3.1. Clearance of βA4 peptides from culture medium of SH-SY5Y cells

Physiological concentrations of synthetic βA4 1-40 or 1-42 were added to the culture medium of SH-SY5Y cells and their catabolism was investigated. When the medium, after culturing for 2–24 h, was withdrawn and analyzed by Western blot assay, we found a marked decrease of βA4 bands in a time-dependent manner (Fig. 1A). Densitometric analysis of the bands revealed that more than 70% of the peptides disappeared after 6 h (Fig. 1B). βA4 produced in vivo (cerebrospinal fluid (CSF) βA4) was also cleared from the medium with a similar time course. When the same amount of βA4 1-40 was added to the cell culture conditioned medium and incubated in the absence of the cells, the peptide was much more stable and about 80% of the peptide remained after 6 h. Also, addition of protease inhibitor mixture (0.3 μM aprotinin, 0.2 μM pepstatin, 0.25 μM leupeptin, 130 μM bestatin and 600 μM phosphoramidon) to the culture medium did not change the cellular clearance rate (data not shown). These results suggest that βA4 clearance is not due to the proteolytic degradation caused by extracellularly released protease.

In order to examine the possibilities that disappeared βA4 is adsorbed on the cell surface or accumulates in intracellular compartments, we next analyzed cell-associated βA4. After 24 h incubation with 50 ng/ml of βA4 1-40, whole cells were lysed and analyzed. Small amounts of peptide were detectable both from the lysate (soluble fraction after detergent lysis) and the pellet (precipitate after detergent treatment and centrifugation) (Fig. 2). However, recovered peptide (about 2 pg from the lysate and 25 pg from pellet) was much less than the total amount of βA4 that had disappeared from the medium (about 5 ng), indicating that the majority of the peptides were degraded, presumably intracellularly after cellular uptake.

3.2. Sequence specificity of βA4 clearance

We next used several different βA4 peptides with N-terminal or C-terminal deletions and compared their clearance. As expected from the results of Fig. 1, only 10–30% of peptide remained when 1-40, 1-42 or CSF βA4 was incubated with cells for 6 h. βA4 1-28 and 17-42 was degraded with similar efficiency. In contrast, βA4 6-40 and 17-40 were found to be much more stable and more than 70% peptide remained after 6 h (Fig. 3).

3.3. Cell type specificity of βA4 clearance

We compared several different cell lines for this βA4 catabolizing activity. Compared to SH-SY5Y cells, incubation with three other cell lines, COS7 (African green monkey epithelial

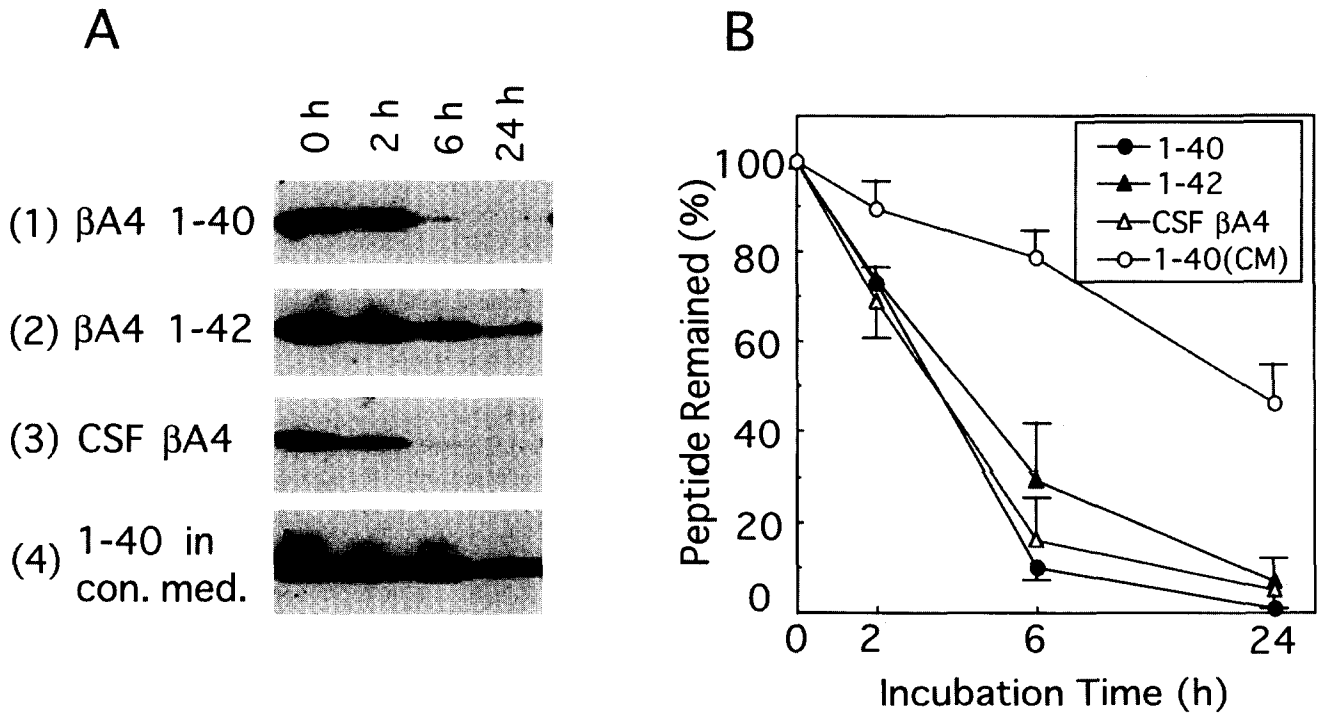

Fig. 1. Clearance of βA4 from SH-SY5Y cell culture medium. A: Western blot detection of βA4. (1) Synthetic βA4 1-40 (50 ng/ml), (2) 1-42 (200 ng/ml) or (3) human CSF was incubated with SH-SY5Y cells in 96-well plate (100 μl/well). After 2, 6, and 24 h, 5 μl medium was withdrawn and analyzed by Western blotting (stained with W0-2 antibody). (4) βA4 1-40 (50 ng/ml) was spiked into the conditioned medium of SH-SY5Y cells (collected after 24 h culture) and analyzed in parallel. When SH-SY5Y cells were cultured in the absence of exogeneous βA4, no band was detectable after 24 h showing endogeneous βA4 production from the cells is negligible (data not shown). B: Time course of βA4 clearance. βA4 band was quantitated by densitometric analysis and expressed as % of initial amount. Values are means (± S.D.) of three assays each.

cells), HeLa (human epithelial cells), and N2a (mouse neuroblastoma), all led to much less prominent clearance of βA4 from the medium (Fig. 4). When we used differentiated SH-SY5Y cells (induced by retinoic acid treatment [14]), we found no difference in the clearance rate from non-treated SH-SY5Y cells (data not shown).

3.4. Stability of intracellularly accumulated peptide

Although most of the βA4 was degraded after uptake by SH-SY5Y cells, a small amount of peptide was detectable intracellularly, as shown in Fig. 2. In order to examine the fate of this internalized peptide in more detail, we incubated the cells with βA4 1-42 peptide for 24 h, replaced the medium by βA4-free fresh medium and analyzed the time-dependent decrease of the intracellular βA4. In accordance with the results of Fig. 2, uptaken peptide was associated with the insoluble cell pellet rather than detergent solubilized fraction, and two closely migrating bands were detected at the position of 4 kDa (the same position as synthetic βA4 1-42) and 3.5 kDa (Fig. 5). Since we used C-terminal specific antibody for the detection here, this lower band presumably represents N-terminally truncated form of βA4. Interestingly, the 4 kDa band decreased time-dependently, while the 3.5 kDa band was more stable and was detectable even after 6 days incubation without βA4 in the medium.

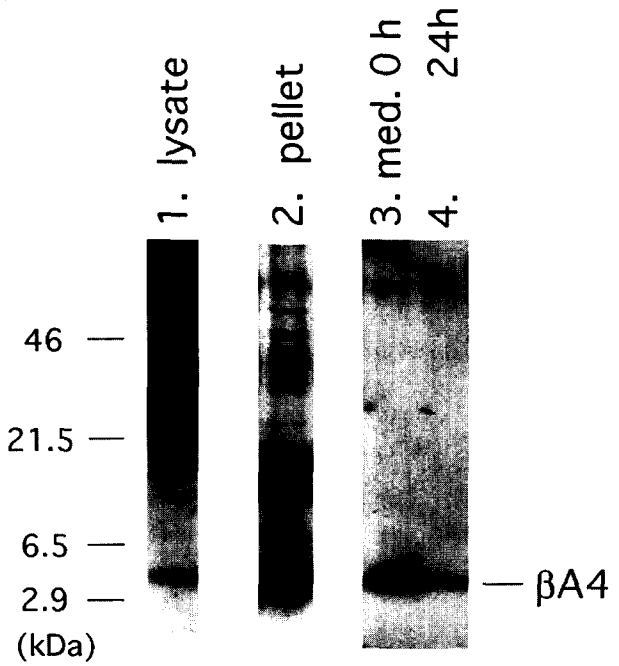

Fig. 2. Analysis of cell-associated βA4. SH-SY5Y cells incubated with synthetic βA4 1-40 (50 ng/ml) for 24 h were lysed and analysed as described in Section 2. Whole sample of immunoprecipitated lysate (lane 1) and pellet (lane 2) (both derived from one well of the cells) was loaded into each lane and analyzed by Western blotting (W0-2 staining). High background of lysate sample (>20 kDa) is derived from antibody used for the immuoprecipitation. Upper bands of pellet sample are non-specific binding of secondary antibody which were also seen when the primary antibody was omitted (data not shown). Aliquots of the medium samples (1 μl medium = 1/500 volume) before (lane 3) and after (lane 4) the incubation were also analyzed.

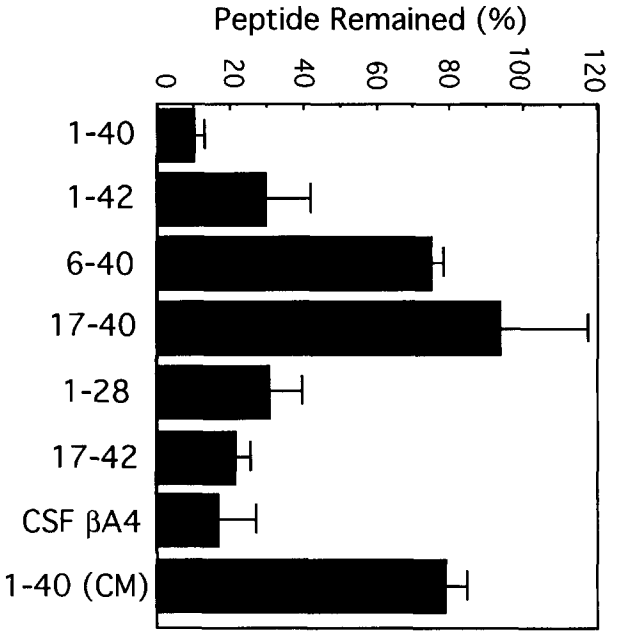

Fig. 3. Peptide sequence specificity of the clearance. Various different βA4-derived peptides (50 ng/ml for 1-40, 6-40 and 17-40; 200 ng/ml for 1-42, 1-28 and 17-42) were incubated with SH-SY5Y cells for 6 h and analyzed. βA4 in CSF sample and βA4 1-40 added to the conditioned medium (without cells) were also analyzed. Values are means (± S.D.) of three assays.

4. Discussion

Since neurons and other cells in the brain are shown to produce soluble βA4 constitutively by normal cell metabolism, there must be a mechanism which catabolizes βA4 and prevents its excessive accumulation. This clearance of βA4 is potentially as important as its secretion from cells in the formation of amyloid plaques.

There exist only a few studies on the βA4 degradation in a cell culture system. Naidu et al. reported that proteinases released by CHO cells transfected with APP gene can degrade βA4 [15]. The activity was inhibited only by the addition of mixed proteinase inhibitors, suggesting that proteinases with several different classes are involved. Another recent report identified a new serine protease complexed with α_2-macroglobulin as a βA4 degrading enzyme, also using APP-transfected CHO cells [16]. In both cases, βA4 was degraded in the conditioned medium (in the absence of cells) by a soluble protease released into the medium. In our study using SH-SY5Y cells, we also detected degradation of βA4 in conditioned medium (Fig. 1). However, we found that βA4 is cleared much more rapidly when incubated in the presence of cells. Together with the observations that extracellularly added protease inhibitors do not block this degradation and that small amounts of peptide can be detected intracellularly, we think the clearance is most likely mediated by a cellular uptake mechanism. Cellular uptake of βA4 has also been reported previously. Ard et al. showed that rat microglia cells can remove βA4 from the medium and accumulate it inside the cells [17]. In that report, however, intracellular accumulation depended on the presence of FCS and the protease inhibitor leupeptin, which was not required in our experiments. In another report, Knauer et al. showed that human skin

fibroblasts accumulate βA4 when cultured with high concentrations of βA4 1-42 [18], although the peptide clearance from the medium was not examined.

Interestingly, we found a sequence specificity of βA4 catabolism by SH-SY5Y cells. While βA4 1-40 and 1-42 disappeared from the medium rapidly, βA4 6-40 and 17-40 were found to be stable. This indicates that the clearance is not due to a simple non-specific degradation of low-molecular weight peptides. Since we also observed cell-type specificity, we speculate that there is a specific interaction between SH-SY5Y cells and βA4. One attractive hypothesis is that SH-SY5Y cells express a cell surface receptor which recognizes βA4, and receptor-mediated endocytosis is involved in the uptake. If this is the case, the receptor is speculated to recognize a conformational state of βA4 rather than its primary sequence, since both βA4 1-28 and 17-42 were degraded while 6-40 and 17-40, which contain the whole overlapping residues of the former two peptides (residues 17-28), were not degraded. In order to examine the possible existence of a receptor, we used higher concentrations of βA4 1-40, which may saturate the binding site. However, we did not observe a decrease of the clearance rate even at the highest concentration (50 μg/ml) (data not shown). Furthermore, degradation of βA4 1-40 (examined with C40 specific antibody) was not inhibited by competing βA4 1-42 with excess amounts (up to 2000-fold molar excess) (data not shown), suggesting that this uptake is not mediated by a high-affinity receptor. The precise molecular mechanism of this uptake-degradation is an important issue to be clarified in future studies.

If this cellular uptake and degradation of βA4 also occurs in vivo in human brain, we think there are two different possible contributions to AD pathogenesis. One simple idea is that this cellular uptake activity prevents the deposition of βA4 by decreasing its extracellular concentration. In this hypothesis, deficient uptake will cause an accumulation of βA4 which leads to its aggregation and deposition. The other possibility is that the intracellular insoluble βA4, which is accumulated through the uptake, serves as a seed for amyloid deposition and promote the plaque formation. By this explanation, enhanced uptake activity will be a cause of disease. In relation to the latter hypothesis, it is highly interesting that several recent biochemical and immunohistochemical analyses showed that the premature 'diffuse' plaques contain considerable amounts of N-terminally truncate βA4 [19–22], which is also generated in this cellular uptake experiment. This intracellular N-terminal processing of βA4 following cellular uptake was also observed by using human skin fibroblast [18].

We assume further studies on this phenomenon will provide a new insight into the mechanism of amyloid formation in vivo, eventually leading to novel therapeutic approaches.

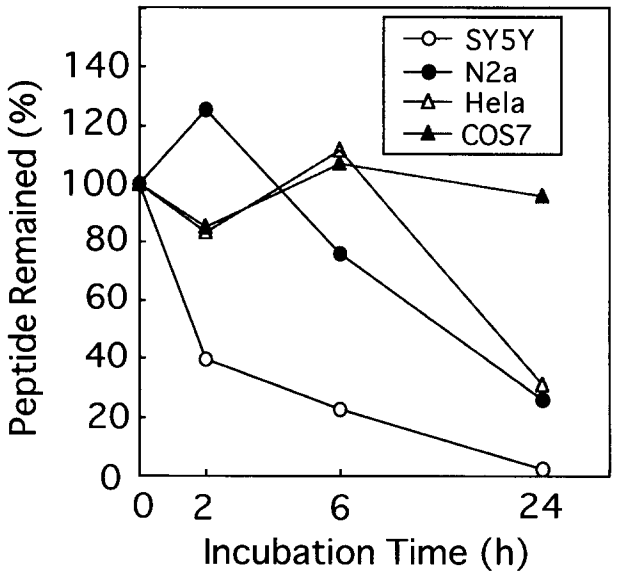

Fig. 4. Cell-type specificity of the clearance. Synthetic βA4 1-40 (50 ng/ml) was incubated with each different cell lines (cultured in 96-well plate) and time-dependent decrease from the medium was measured. Values are means of two assays.

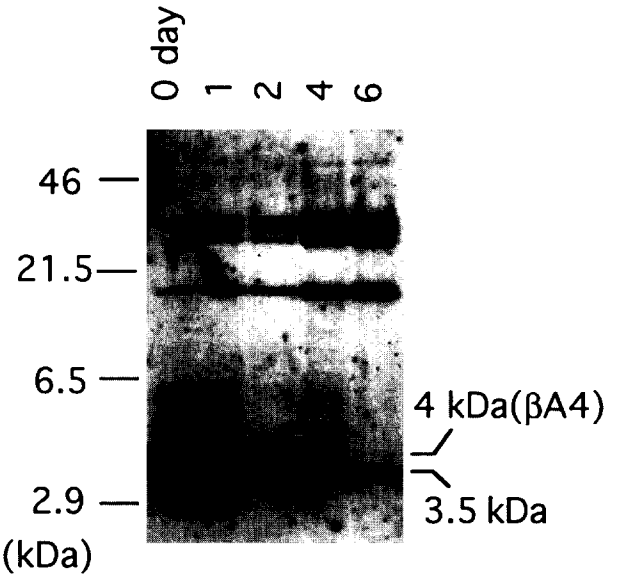

Fig. 5. Stability of intracellularly accumulated βA4. SH-SY5Y cells cultured with synthetic βA4 1-42 (50 μg/ml) were further cultured in the absence of βA4 and intracellular βA4 was analyzed by Western blotting using C-terminus specific antibody (clone G2-11). Day 0 represents the cells analyzed immediately after the 24 h incubation with βA4 and washing. Only the pellet samples are shown, since βA4 band was not detectable from lysate samples in this assay.

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