Heliyon

Received:
31 October 2017
Revised:
5 January 2018
Accepted:
8 January 2018

Cite as: Yoichi Ohshima, Katsutoshi Taguchi, Ikuko Mizuta, Masaki Tanaka, Takami Tomiyama, Fuyuki Kametani, Chihiro Yabe-Nishimura, Toshiki Mizuno, Takahiko Tokuda. Mutations in the β-amyloid precursor protein in familial Alzheimer's disease increase Aβ oligomer production in cellular models. Heliyon 4 (2018) e00511.
doi: 10.1016/j.heliyon.2018.e00511

Mutations in the β-amyloid precursor protein in familial Alzheimer's disease increase Aβ oligomer production in cellular models

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Abstract

Soluble oligomers of amyloid-β (Aβ) peptides (AβOs) contribute to neurotoxicity in Alzheimer's disease (AD). However, it currently remains unknown whether an increase in AβOs is the common phenotype in cellular and animal models. Furthermore, it has not yet been established whether experimental studies

http://dx.doi.org/10.1016/j.heliyon.2018.e00511

conducted using models overexpressing mutant genes of the amyloid precursor protein (APP) are suitable for investigating the underlying molecular mechanism of AD. We herein employed the Flp-In^{TM} T-REx^{TM}-293 (T-REx 293) cellular system transfected with a single copy of wild-type, Swedish-, Dutch-, or London-type *APP*, and quantified the levels of Aβ monomers (Aβ1-40 and Aβ1-42) and AβOs using an enzyme-linked immunosorbent assay (ELISA). The levels of extracellular AβOs were significantly higher in Dutch- and London-type *APP*-transfected cells than in wild-type *APP*-transfected cells. Increased levels were also observed in Swedish-type *APP*-transfected cells. On the other hand, intracellular levels of AβOs were unaltered among wild-type and mutant *APP*-transfected cells. Intracellular levels of Aβ monomers were undetectable, and no common abnormality was observed in their extracellular levels or ratios (Aβ1-42/Aβ1-40) among the cells examined. We herein demonstrated that increased levels of extracellular AβOs are the common phenotype in cellular models harboring different types of *APP* mutations. Our results suggest that extracellular AβOs play a key role in the pathogenesis of AD.

Keyword: Neuroscience

1. Introduction

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder and is characterized pathologically by the emergence of senile plaques and neurofibrillary tangles (NFTs) in the brain. The former and latter are extracellular and intracellular protein aggregates composed of amyloid β (Aβ) and hyperphosphorylated tau protein, respectively. Previous studies demonstrated that the accumulation of Aβ precedes and triggers the hyperphosphorylation of tau [1, 16, 20, 21, 30]. Genetic studies linked early-onset familial AD (FAD) to various mutations in the genes encoding β-amyloid precursor protein (APP) as well as presenilin 1 and 2 (PS1 and PS2), and most of these mutations share a common phenotype by showing an absolute or relative increase in the production of the highly fibrillogenic Aβ1-42 peptide [25, 26]. Based on these findings, the "amyloid cascade hypothesis" has been widely accepted as the centerpiece of AD pathogenesis, and insoluble Aβ amyloid fibrils were originally regarded as the primary neurotoxic molecules for AD [8, 27]. However, recent studies showed that cognitive impairment in patients with AD correlated with the amount of soluble Aβ oligomers (AβOs) rather than that of insoluble Aβ fibrils [7, 12]. Furthermore, increasing evidence has shown that AβOs cause neurotoxicity and cognitive impairment not only in experimental models [6, 14, 32], but also in humans [13, 15, 19, 28]. These findings strongly suggest that soluble AβOs, not Aβ fibrils, play a central role in the pathogenesis of AD [7, 17, 24]. In a previous study using induced pluripotent stem cells (iPSCs) from patients with FAD, intracellular AβOs were shown to be increased in iPSC-derived neurons carrying the Osaka (E693Δ)-type *APP* mutation, but not in those

http://dx.doi.org/10.1016/j.heliyon.2018.e00511

carrying the Indiana (V717L)-type *APP* mutation [11]. However, it is still unclear whether an increase in intracellular or extracellular AβOs is a common phenotype in cellular models carrying various gene mutations linked to FAD.

Experimental studies on AD have largely been conducted using cellular and animal models overexpressing the mutant genes of *APP*. However, these models represent artificial phenotypes because they overproduce not only Aβ peptides, but also APP and its fragments [18, 22, 23]. Moreover, cellular models that overexpress mutant *APP* may not be suitable for examining and comparing the production levels of AβOs among cell lines harboring various FAD mutations. In order to overcome these issues, we used a cellular system stably transfected with a single copy of wild-type or mutant *APP*.

Regarding the quantification of AβOs, we previously developed an original enzyme-linked immunosorbent assay (ELISA) system that specifically detects high-molecular-weight (HMW) AβOs mainly composed of 45- to 90-kDa oligomers (10–20 mers) [5, 9, 10]. Our AβO-ELISA detects Aβ dodecamers (referred to as Aβ*56), which have been correlated with memory deficits in APP transgenic Tg2576 mice [15]. The levels of HMW-AβOs determined by AβO-ELISA in the cerebrospinal fluid (CSF) were significantly higher in patients with AD or mild cognitive impairment than in age-matched controls [5]. In the present study, we examined the levels of extracellular and intracellular Aβ species including monomeric Aβs and AβOs in cells transfected with a single copy of wild-type or mutant *APP* alleles linked to FAD. We herein demonstrated that increased levels of extracellular AβOs are the common phenotype in mutant *APP*-transfected cells.

2. Materials and methods

2.1. Reagents

We purchased the following antibodies and reagents from the respective manufacturers listed below. A mouse monoclonal antibody to Aβ (6E10) (SIG-39300, Covance, CA, USA); a rabbit polyclonal antibody to APP, C-terminal (A8717, Sigma-Aldrich, MO, USA); a β-actin rabbit antibody (#4970, Cell signaling, MA, USA); a goat anti-mouse IgG-horseradish peroxidase (HRP) antibody (sc-2005, Santa Cruz Biotechnology, CA, USA); a goat anti-rabbit IgG-HRP antibody (sc-2054, Santa Cruz Biotechnology); Alexa Fluor 488 Goat anti-rabbit IgG (H + L) (A11088, Life Technologies, CA, USA); Human β Amyloid (1–40) ELISA kit (292-62301, Wako, Osaka, Japan); Human β Amyloid (1–42) ELISA kit (298-62401, Wako); Protein G-Agarose (11719416001, Roche, Basel, Switzerland); hygromycin B (400052, Calbiochem, CA, USA); SuperSignal West Femto Maximum Sensitivity Substrate (#34095, Thermo Fisher Scientific, Rockford, USA); SuperSignal ELISA Femto Maximum Sensitivity Substrate

http://dx.doi.org/10.1016/j.heliyon.2018.e00511

(#37075, Thermo Fisher Scientific); Ham's F-12 medium (17458-65, Nacalai Tesque, Kyoto, Japan); Penicillin-Streptomycin (15140-122, Gibco, CA, USA); and fetal bovine serum (SH30910.03, Hyclone, MA, USA).

2.2. Vector construction

In order to introduce human wild-type *APP*695 cDNA and Swedish-mutant *APP*695 cDNA (K670NM671L) into a pcDNA5/FRT/TO vector (Invitrogen), pEF-BOS vectors harboring wild- or Swedish-type *APP* (gifted from Dementia and Higher Brain Function Research, Tokyo Metropolitan Institute of Medical Science) were used as templates to amplify *APP* cDNA with or without mutations by PCR. Using the Rapid DNA Ligation Kit (Roche), PCR products were inserted into the multi-cloning sites of pcDNA5/FRT/TO. The Dutch (E693Q) and London (V717I) mutations were introduced into the wild-type *APP* plasmid by site-directed mutagenesis using appropriate oligonucleotides and the KOD-Plus-Mutagenesis Kit (TOYOBO). The control plasmid vector and vectors harboring human wild-, Swedish-, Dutch-, or London-type *APP* were sequenced for confirmation of each mutation, and transfected into Flp-In™ T-REx™-293 (T-REx 293, Invitrogen, CA, USA) cells.

2.3. Cell culture and transfection

T-REx 293 cells were cultured in Ham's F-12 medium containing 10% fetal bovine serum. Cells were co-transfected with a pOG44 vector (Invitrogen) and pcDNA5/FRT/TO vector coding for wild-type or mutant *APP* with lipofectamine LTX as described [33]. Cells transfected with the empty pcDNA5/FRT/TO vector were used as a negative control. Stable cell lines were selected in the presence of 100 μg/ml hygromycin B. In order to express exogenous *APP*, T-REx293 cell lines were incubated with 1 μg/ml tetracycline (Tet-On system).

2.4. Immunostaining

Cells were cultured in 24-well plates with Ham's F-12 medium containing tetracycline at 37 °C for two days and fixed with 2% paraformaldehyde containing phosphate buffered saline (PBS, Dainippon Sumitomo, Osaka, Japan) as described [33]. Cells were incubated for two hours with an anti-APP-C antibody (1:1000), followed with Alexa Fluor 488 Goat anti-rabbit IgG (1:1000) for one hour at room temperature. The expression of APP was observed using confocal laser scanning microscopy (FV1000, OLYMPUS, Tokyo, Japan).

2.5. Sample preparation for immunoblotting and ELISA

Cells were cultured in serum-free Ham's F-12 medium containing tetracycline in 10-cm dishes at 37 °C for two days. Culture media were collected with a protease

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inhibitor cocktail (04080-11, Nacalai Tesque) and cells were dissolved in RIPA buffer (50 mM Tris-HCl buffer pH 7.6, 150 mM NaCl, 1% Nonidet P40, 0.5% Sodium Deoxycholate) containing a protease inhibitor (08714–04, Nacalai Tesque). Following centrifugation, the protein amount in the supernatant fraction was determined using a BCA protein assay kit (Thermo Fisher Scientific).

2.6. Immunoblotting

In order to detect extracellular Aβ monomers or AβOs, those molecules produced by each cell line were immunoprecipitated. An anti-Aβ antibody (6E10) was added to the supernatants and immune complexes were recovered with protein G-agarose beads. Following their elution with 20 μl lithium dodecyl sulfate (LDS) sample loading buffer (Invitrogen), immune complexes were loaded on 12% Bis-Tris Protein Gels (Invitrogen), and transferred to PVDF membranes (0.45 μm, Millipore). These membranes were pretreated with Blocking One (Nacalai Tesque) at room temperature and incubated with an anti-APP-C, anti-β-actin, or anti-Aβ (6E10) antibody. Following the incubation with a goat anti-mouse or rabbit IgG −HRP antibody at room temperature, the membranes were treated with chemiluminescent reagents, and proteins were detected using ImageQuant LAS 4000 mini (GE Healthcare, Buckinghamshire, UK). APP levels were quantified by measuring the pixel density of the band with ImageJ software (NIH). The summed density of the two bands between 110–160 kDa was calculated and evaluated as the amount of total APP. The levels of Aβ monomers and oligomers measured by ELISA were corrected by each amount of total APP.

2.7. ELISAs for monomeric Aβ species and AβOs

Aβ1-40 and Aβ1-42 levels in culture media were measured using the Human β Amyloid (1–40) ELISA kit (Wako) and Human β Amyloid (1–42) ELISA kit (Wako), respectively, in accordance with the manufacturer's instructions. The ELISA reaction was developed with 3,3′,5,5′-Tetramethylbenzidine (TMB) solution in the kit.

The levels of AβOs in culture media and cell lysates were measured using our AβOs-specific ELISA system [5, 10]. In brief, 96-well plates were incubated with the carbonate buffer containing the anti-Aβ monoclonal antibody, BAN50 (10 μg/ml) at 4 °C overnight. Culture media, cell lysates, or standard reagents containing AβOs were applied to each well and incubated at 4 °C overnight. The detector antibody, the HRP-conjugated Fab′ fragment of BAN50 diluted 1:2500 with buffer C (heat-inactivated 20 mM phosphate buffer, pH 7.0, containing 0.2% protease-free BSA, 2 mM EDTA, 400 mM NaCl, and 0.05% merthiorate Na) was added to the wells, and incubated at room temperature for 3 h. The chemiluminescent

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substrates (SuperSignal ELISA Femto Maximum Sensitivity Substrate) were then added to the wells.

The enzymatic products were measured using a microplate spectrophotometer (SpectraMax Plus384; Molecular Devices, Osaka, Japan) at 450 nm (OD_{450}) for the TMB substrate or with a luminometer (SpectraMaxL; Molecular Devices, Osaka, Japan) for the chemiluminescent substrate. The levels of Aβ1-40, Aβ1-42, and AβOs were corrected by the level of total APP quantified as described above (2.6) in the cell lysates of each experiment. In order to compare the levels of Aβ monomers and AβOs among different cell lines, they were expressed as ratios against the amount of each molecule produced by wild-type *APP*-transfected cells in simultaneously performed experiments.

2.8. Statistical analysis

Values are given as means ± SEM. All data was analyzed using a one-way ANOVA followed by Dunnett's test with GraphPad Prism6 (GraphPad Software, CA, USA).

3. Results

3.1. Expression of APP in transfected cell lines

Each cell line expressing exogenous *APP* with or without mutations was immunostained by the anti-APP-C antibody (Fig. 1A). No significant differences were observed in the intracellular distribution of APP among the cell lines examined. In order to confirm the expression levels of APP in T-REx 293 cells, we quantified the amount of total APP in the cell lysates. Immunoblot analyses revealed that a similar amount of APP was expressed in these cell lines (Fig. 1B, C).

3.2. Detection of Aβ monomer species in *APP*-transfected cells

Immunoblotting of culture media demonstrated Aβ monomer bands in all *APP*-transfected cell lines around 4-kDa (Fig. 2A). However, there was no band corresponding to Aβ monomers in the cell lysates (data not shown).

The levels of Aβ monomers in culture media were then quantified by ELISA specific for Aβ monomers. The extracellular levels of Aβ1-40 and Aβ1-42 were significantly higher in Swedish-type *APP*-transfected cells than in wild-type *APP*-transfected cells ($p < 0.05$, n = 5), but were not altered in Dutch-type *APP*-transfected cells (Fig. 2B, C). In London-type *APP*-transfected cells, the extracellular level of Aβ1-40 was similar to that of control APP cells, whereas the level of Aβ1-42 was significantly higher ($p < 0.05$, n = 5). Therefore, the ratio of extracellular Aβ1-42 to Aβ1-40 was significantly increased in London-type

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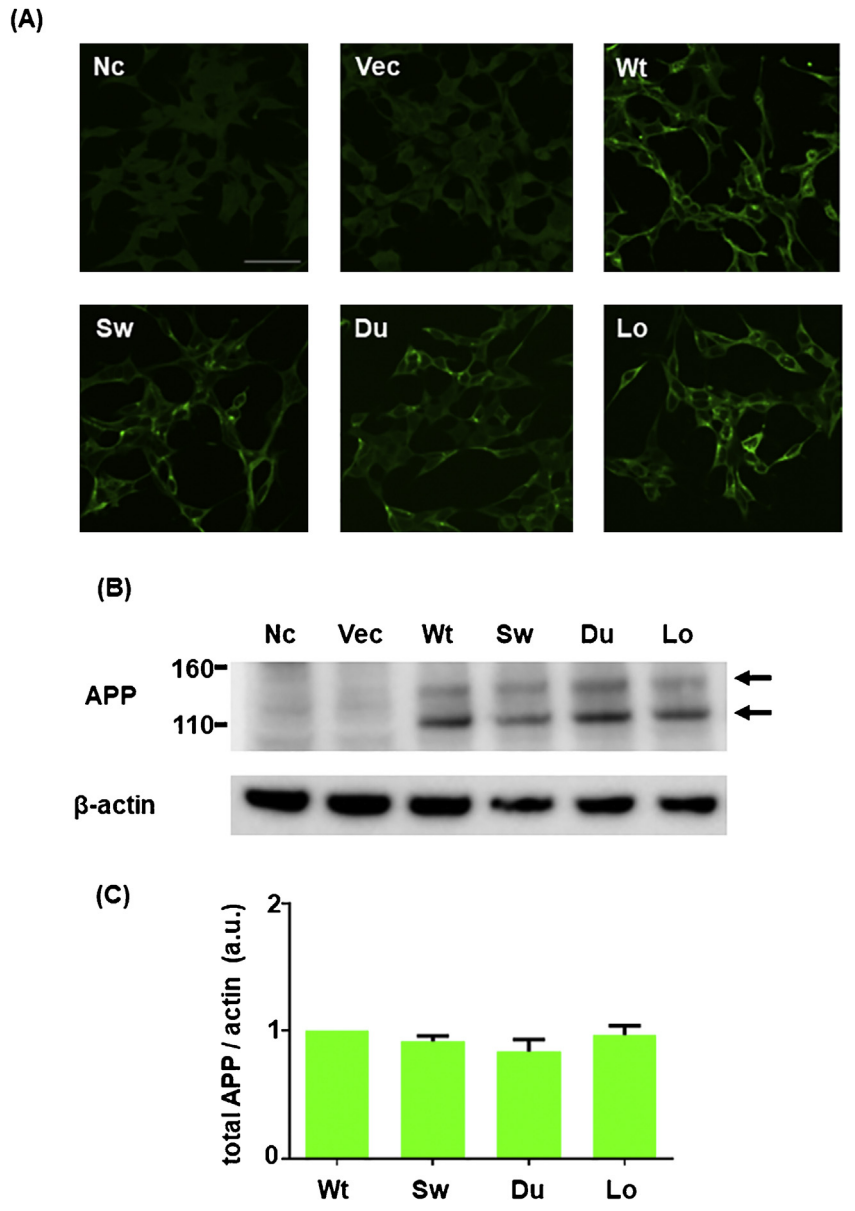

Fig. 1. Expression of APP in wild-type or mutant *APP*-transfected cells. (A) The distribution of APP was similar in *APP*-transfected cells. The scale bar represents 50 μm. (B) The expression of total APP and β-actin in cell lysates (5 μg protein/lane) was detected by immunoblotting. Full-sized images are available in Supplementary Material. The summed density of the two bands (arrows) was quantified as the amount of total APP and demonstrated in (C). Nc: negative control, Vec: pcDNA5/FRT/TO vector-transfected cells, Wt: wild-type, Sw: Swedish, Du: Dutch, Lo: London-type mutant-transfected cells, respectively, a.u.: arbitrary unit.

APP-transfected cells ($p < 0.05$, n = 5). This ratio was slightly lower in Swedish-type *APP*-transfected cells than in control APP cells, but was unaltered in Dutch-type *APP*-transfected cells (Fig. 2D). Aβ monomers were not detected in cell lysates by ELISA, similar to the results obtained by immunoblot analyses.

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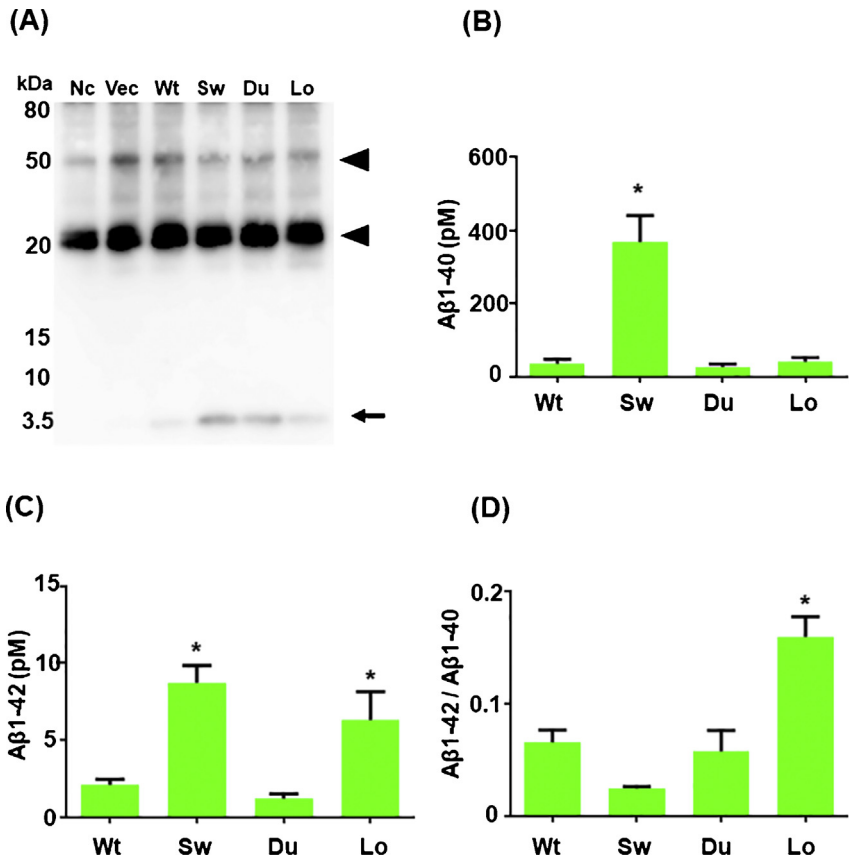

Fig. 2. Extracellular levels of Aβ monomers in *APP*-transfected cells. (A) Aβ monomers (arrow) were detected in the culture media of cells transfected with wild-type or mutant *APP*. Bands corresponding to high-molecular-weight (HMW) AβOs or low-molecular-weight (LMW) AβOs were not detected. Arrowheads indicate the immunoglobulin light chain and heavy chain. (B) The levels of Aβ1-40 and (C) Aβ1-42 in culture media determined by ELISA. (D) The ratio of Aβ 1–42 to Aβ 1–40 present in the culture media of *APP*-transfected cells. Data are the means ± SEM. n = 5 independent experiments, one-way ANOVA followed by Dunnett's test; $*p < 0.05$.

3.3. Detection of AβOs in *APP*-transfected cells

Extracellular and intracellular AβOs were not detected in *APP*-transfected cells by immunoblotting. However, when the amount of AβOs was analyzed using our AβO-specific ELISA system, extracellular and intracellular AβOs were readily detected. As shown in Fig. 3A, the levels of extracellular AβOs were significantly higher in the culture media of Dutch ($p < 0.05$, n = 5) and London-type *APP*-transfected cells ($p < 0.01$, n = 5) than in that of control APP cells. The levels of AβOs also tended to be higher in Swedish-type *APP*-transfected cells ($p = 0.09$, n = 5).

Intracellular levels of AβOs in *APP*-transfected cells were determined by AβO-specific ELISA. These levels were similar ($p = 0.94$, n = 5) among *APP*-transfected cells (Fig. 3B). Therefore, increased levels of extracellular, but not

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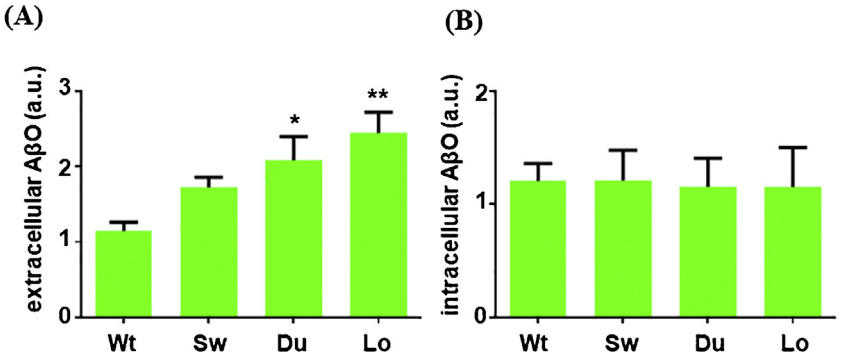

Fig. 3. Extracellular and intracellular levels of AβOs in *APP*-transfected cells. (A) The levels of AβOs in culture media determined by AβO-specific ELISA. (B) The levels of AβOs in cell lysates determined by AβO-specific ELISA. Data are the means ± SEM. n = 5 independent experiments, one-way ANOVA followed by Dunnett's test; $*p < 0.05$, $**p < 0.01$.

intracellular AβOs appear to be the common phenotype in the Dutch- and London-type *APP*-transfected cells.

4. Discussion

We herein demonstrated that the levels of extracellular AβOs significantly increased in Dutch and London-type *APP*-transfected cells, and tended to be higher in the three cell lines we examined. Meanwhile, there was no common abnormality in the extracellular levels or ratios of Aβ1-40 and Aβ1-42. The levels of intracellular AβOs were equivalent to those in wild-type *APP*-transfected cells. Hence, increased levels of extracellular AβOs appear to be the common pathological phenotype in cellular models carrying *APP* mutations linked to FAD (Table 1).

Table 1. The levels of extracellular or intracellular Aβ species in wild-type or mutant *APP*-transfected cells.

Aβ		Swedish	Dutch	London
monomer	Aβ1-40	↑↑	→	→
	Aβ1-42	↑↑	→	↑↑
	Aβ42/Aβ40	↓	→	↑↑
oligomer	extracellular	↑	↑↑	↑↑
	intracellular	→	→	→

The levels of Aβ1-40 and Aβ1-42 increased in culture media secreted from cells with Swedish-type *APP*, and the ratio of Aβ1-42 to Aβ1-40 increased in those with London-type *APP*. The levels of AβOs significantly increased in culture media from Dutch- and London-type *APP*-transfected cells, and tended to increase in culture media from Swedish-type *APP*-transfected cells compared with those from wild-type *APP*-transfected cells. The levels of intracellular AβOs in mutant *APP*-transfected cells were not different from those of wild-type *APP*-transfected cells.

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Previous studies were conducted using cellular and animal models that overexpress *APP* in order to quantify the amount of Aβ species relevant to the development of AD. However, the phenotypes demonstrated in mice overexpressing human *APP* were found to be artifacts resulting from high levels of APP and its non-Aβ fragments [22]. Accordingly, concerns have been raised regarding these overexpression models. In order to avoid these issues, we used a cellular system expressing a single copy of *APP*, and compared the levels of Aβ monomers and AβOs among wild-type and mutant *APP*-transfected cells under more physiological conditions.

In this study, the levels of extracellular AβOs were higher in mutant *APP*-transfected cells. It has been unclear in previous studies whether the levels of AβOs increase or not among cellular models transfected with different types of *APP* mutations. AβOs are heterogeneous assemblies that widely range in size [2]. The sizes of AβOs, which are the most neurotoxic species leading to AD, remain controversial. Aβ dimers from the brains of patients with AD were found to inhibit long-term potentiation in the hippocampus of normal mice and disrupted memory in normal rats [28]. Furthermore, the 56-kDa Aβ dodecamers (Aβ*56) purified from APP-transgenic mice disrupted memory in young rats [15]. Our ELISA system specific to HMW-AβOs (45- to 90-kDa oligomers) detects these AβOs including Aβ*56. Therefore, our results suggest that HMW-AβOs play a crucial role in the pathogenesis of AD.

The intracellular accumulation of AβOs was previously reported in neurons that differentiated from iPSCs prepared from a sporadic or Osaka-type FAD patient [11]. However, in the present study, the levels of intracellular AβOs were similar among wild-type and mutant *APP*-transfected cell lines. The accumulation of AβOs was not observed in neurons that differentiated from iPSCs prepared from another sporadic or Indiana-type FAD patient [11]. Therefore, it remains unclear whether AβOs general neurons (extracellular Aβ monomers) is the pivotal abnormality leading to the development of AD, based on the findings obtained from cellular models overexpressing mutant *APP* linked to FAD [25, 26]. However, the extracellular levels or ratios of Aβ1-42 and Aβ1-40 produced from our cellular models expressing a single copy of FAD-linked mutant *APP* largely varied according to the types of *APP* mutations. In previous studies on the London-type *APP* mutation, the total amount of Aβ was unchanged or decreased, while the ratio of Aβ1-42 to Aβ1-40 increased [4] or decreased [29]. Other studies demonstrated that the production of Aβ1-42 and Aβ1-40 in Swedish-type *APP*-transfected cells markedly increased [3], whereas the ratio of Aβ1-42 to Aβ1-40 significantly decreased [31]. Furthermore, Aβ monomers in cell lysates (intracellular Aβ monomers) have not yet been successfully detected by immunoblotting or ELISA in the present experiments. This may be due to the rapid release of Aβ monomers into culture media once produced by the cleavage of APP on cellular membranes.

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5. Conclusions

Increased levels of HMW-AβOs were shown to be the common phenotype of cellular models expressing Swedish-, Dutch-, and London-type mutant *APP*. Our results suggest that extracellular HMW-AβOs produced from cerebral neurons play a central role in the pathogenesis of AD, and may be an important target of disease-modifying therapies.

Declarations

Author contribution statement

Yoichi Ohshima, Katsutoshi Taguchi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ikuko Mizuta, Takami Tomiyama, Fuyuki Kametani: Contributed reagents, materials, analysis tools or data.

Masaki Tanaka: Conceived and designed the experiments; Analyzed and interpreted the data.

Chihiro Yabe-Nishimura, Toshiki Mizuno: Conceived and designed the experiments.

Takahiko Tokuda: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Competing interest statement

The authors declare no conflict of interest.

Funding statement

This work was supported by JSPS KAKENHI Grant Number JP25461288 (YO).

Additional information

Supplementary content related to this article has been published online at 10.1016/j.heliyon.2018.e00511.

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