Observation of amyloid precursor protein cleavage and A β generation in living cells by using multiphoton laser scanning microscopy

Xiao-Qing LI¹, Su-Ming ZHANG¹, Hua-Jing YANG¹, Zhi-Hong ZHANG²

¹Department of Neurology, Tongji Hospital, Tongji Medical College, ²The Key Laboratory of Biomedical Photonics of Ministry of Education, Huazhong University of Science and Technology, Wuhan 430030, China

Abstract: Objective To investigate the proteolytic mechanism of amyloid precursor protein (APP) and to explore amyloid-beta (A β) generation in living neurons. **Methods** DNA fragments were amplified by PCR or synthesized. The four fragments, CFP, 54bp, YFP and C99 were ligated into pcDNA3.0 vector to construct the recombinant plasmids pcDNA3.0-CFP-54bp-YFP and pcDNA3.0-CFP-54bp-YFP-C99. The SH-SY5Y cells were transiently transfected with pcDNA3.0-CFP-54bp-YFP or pcDNA3.0-CFP-54bp-YFP-C99. The expression of fusion gene was examined under a multiphoton laser scanning microscope. Fluorescence resonance energy transfer (FRET) was used to measure the β cleavage and γ cleavage of APP. A β generation was confirmed by immunocytochemistry and multiphoton laser scanning microscopy. Cell viability was tested by MTT assay at different time points. **Results** (1) The double restriction endonuclease digestion and sequencing analysis confirmed the authenticity of the recombinant plasmids pcDNA3.0-CFP-54bp-YFP and pcDNA3.0-CFP-54bp-YFP-C99. (2) Blue and yellow fluorescences were detected in the transfected cells. (3) FRET occurred in pcDNA3.0-CFP-54bp-YFP-transfected cells but not in pcDNA3.0-CFP-54bp-YFP-C99-transfected cells. (4) A β was produced in the pcDNA3.0-CFP-54bp-YFP-C99 transfected cells. (5) A β -deposition was widespread in the cell. (6) Cell viability decreased along with the intracellular A β deposition. **Conclusion** C99 is important for the APP β cleavage. A β may be generated and deposited in cells at the early stage of Alzheimer's disease. Intracellular A β accumulation brings deleterious effects on cells.

Keywords: amyloid precursor protein; amyloid beta protein; beta-cleavage; fluorescence resonance energy transfer

1 Introduction

Alzheimer's disease (AD) is a neurological degenerative disease and one of its characteristic pathological changes is senile plaque, the main component of which is Amyloid beta (A β) protein^[1-6]. However, the biochemical mechanisms and the cellular compartments involved in A β production have not been fully elucidated yet.

Fluorescence resonance energy transfer (FRET), the nonradiative transfer of photon energy from an excited fluorophore (donor) to another fluorophore (acceptor), occurs only when the donor and acceptor are in close proximity (10-100 Å). Therefore, this approach can be used to

measure a nanometer scale distance and has high resolution. FRET is used to detect protein-protein interaction and proteolytic process in living cells. Cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) can label two proteins to detect the intermolecular interaction, or label two termini of a protein to detect the intramolecular domain interaction or proteolysis in living cells^[7,8].

In the present study, we constructed two recombinant plasmids, which separately encode an amyloid precursor protein (APP) mutant protein and a fluorescent protein, to explore the mechanisms of A β production and the proteolytic process of APP.

2 Materials and methods

2.1 Fragment preparation and construction of recombinant plasmid The first fragment encoding the cyan fluorescent protein was named as CFP. The second fragment with 54 bases was named as 54bp which encoded an 18-aa

Corresponding author: Su-Ming ZHANG
Tel: 86-27-83612633
E-mail: suming_zhang@163.com
Article ID: 1673-7067(2007)05-0256-07
CLC number: R742
Document code: A
Received date: 2007-03-29

peptide containing the Swedish mutation of APP. The third fragment encoding the yellow fluorescent protein was named as YFP. The fourth fragment encoding the last 99 aa of the APP protein was named as C99, which harbored APP717 mutation. The recombinant plasmid was named as pcDNA3.0-CFP-54bp-YFP-C99.

For generation and ligation of the four fragments CFP, YFP, 54bp and C99, the plasmid pcDNA3.0-CFP-CaM-YFP (provided by Professor Zhi-Hong Zhang), which contained two *Bgl*II sites, was digested by the restriction endonuclease *Bgl*II. The pcDNA3.0-CFP-CaM-YFP was thus split into two fractions. The larger fraction was the PCR template for CFP and the smaller one was for YFP. CFP was amplified by PCR with the upstream primer 5'-AGG CGT GTACGGTGGGAGGTCTA-3' and the downstream primer 5'-TATGGGATCCGGCGGCG-3'. The reaction was carried out under the condition of 94°C for 5 min, 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (for 30 cycles), followed by an additional extension at 72°C for 5 min. *Hind*III and *Bam*HI sites were designed at two ends respectively of the PCRred CFP fragment. YFP was amplified by PCR with the upstream primer 5'-ATT TCT CGA GAT GGT GAG CAA GGG-3' and the downstream primer 5'-CAG AAT CTA GAC TTG TAC AGC TCG TCC AT-3'. The reaction was carried out under the condition of 94°C for 5 min, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min (for 30 cycles) followed by an additional extension at 72°C for 5 min. *Xho*I and *Xba*I sites were introduced onto two ends respectively of the PCRred YFP fragment. The fragment 54bp was synthesized by the biological company and its upstream sequence was 5'-GAT CCA AGA CGG AGG AGA TCT CTG AAG TGA ATC TGG ATG CAG AAT TCC GAC ATG ACT CAC-3' and the downstream sequence was 5'-TCG AGT GAG TCA TGT CGG AAT TCT GCA TCC AGA TTC ACT TCA GAG ATC TCC TCC GTC TTG-3'. *Bam*HI and *Xho*I sites were introduced onto two termini respectively of the 54bp fragment. Finally the fragment C99 was produced by PCR the APP717 mutation-harbored plasmid pcDNA3.0-APP. The primers were 5'-AGC TCT CTA GAG ATG CAG AAT TCC GAC A-3' and 5'-CGT CCA GCG GGC CCC TAG T-3'. The reaction was carried out under the condition of 94°C for 5 min, 94°C for 1 min, 58°C for 1 min and 72°C for 1 min (for 36 cycles) followed by an additional extension at 72°C for 5 min. The PCRred C99 fragment contained *Xba*I and *Apa*I sites at two ends of the fragment, respectively. The fragment CFP was subcloned into the *Hind*III and *Bam*HI sites of the eukary-

otic expression vector pcDNA3.0; the 54bp was into the *Bam*HI and *Xho*I sites; the YFP was into the *Xho*I and *Xba*I sites; and the C99 was into the *Xba*I and *Apa*I sites.

To confirm the construction of the recombinant plasmids, two approaches were used to detect the sequence. First, the construct was identified by the restriction digestion analysis. For the recombinant plasmid pcDNA3.0-CFP-54bp-YFP-C99, we carried out different double digestions including *Hind*III-*Bam*HI, *Xho*I-*Xba*I *Xba*I-*Apa*I and *Hind*III-*Apa*I, which resulted in the release of fragment CFP, YFP, C99 and CFP-54bp-YFP-C99, respectively. Second, the constructs were confirmed by sequencing analysis.

2.2 Cell culture and transfection Human neuroblastoma SH-SY5Y cells were cultured in the Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. When reaching 80% confluent, SH-SY5Y cells were transiently transfected with the recombinant plasmids motioned above by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacture's instructions and cultured in serum-free medium.

2.3 Imaging FRET by multiphoton laser scanning microscopy The technical support was provided by the associates in the Key Laboratory of Biomedical Photonics of Ministry of Education, Huazhong University of Science and Technology, China. Multiphoton laser scanning imaging system included Laser Scanning Confocal Microscopy MRC-1024MP (Bio-Rad Ltd.), NIKON inverted microscope (TE300), Sapphire Femtosecond Laser (TSUNAMI), and Ar⁺ Laser (American Laser Corporation).

The emission spectrum of CFP (460-520 nm) overlaps with the excitation spectrum of YFP (480-520 nm), thus FRET can be measured by spectrum confocal microscopy in living cells. If CFP and YFP are in close proximity (<100 Å), part of the emission energy under CFP excitation will transferred from CFP to YFP, therefore increase the YFP emission and decrease the CFP emission.

At different times after transfection, the transfected cells were illuminated with laser and examined with a microscope. The CFP was excited at 433 nm and the images were acquired through photo multiplier tube 1 (PMT1) at the emission wavelength of 465-512 nm. The FRET images were acquired through PMT2 at the emission wavelength of YFP (543-618 nm).

2.4 Immunocytochemistry A β generation in the cells transfected with pcDNA3.0-CFP-54bp-YFP-C99 was detected by immunocytochemistry. The transfected cells were fixed,

washed and permeabilized, then blocked with normal goat serum before incubated with primary antibodies (1:50 dilution) against A β at 4 °C for 36 h. After washing, the secondary antibodies (1:100 dilution) conjugated with cyanine3 (Cy3) were applied at room temperature for 2 h. Finally, the 4,6-diamidino-2-phenylindole1 (DAPI) nuclear staining was done.

2.5 Methyl thiazol trtrazolium (MTT) assay Cells were divided into 3 groups: control group, CFP-54bp-YFP group, and CFP-54bp-YFP-C99 group. Two hundreds microlitre of cell suspensions in each group was added into different wells of a 96-well plate. Cell viability was measured by MTT assay at different time points (12 h, 24 h, 36 h, 48 h, 72 h and 96 h) post-transfection. The MTT stock solution (5 mg/mL) was added to the growing cultures with a final concentration at 0.5 mg/mL and the mixture was incubated for 4 h. The medium was removed, then 150 μ L DMSO was added to the cells and vortexed. Optical density was measured at 490 nm with a spectrophotometer (SPECTRA-max PLUS, Molecular Devices). Three replicates were determined for each sample and the curve was depicted. Data were analyzed by *t*-test and expressed as mean $\pm$ SEM. *P* < 0.05 was considered statistically significant.

3 Results

3.1 Construction of recombinant plasmid The restriction endonuclease digestion and sequencing analysis confirmed

Fig. 1 Identification of the recombinant plasmid pcDNA3.0-CFP-54bp-YFP-C99 by different double-endonuclease digestions. Lane M: DNA marker. Lane A: pcDNA3.0-CFP-54bp-YFP-C99 double-digested by *HindIII*-*BamHI*. The arrow indicates the fragment CFP, about 702 bp. Lane B: pcDNA3.0-CFP-54bp-YFP-C99 double-digested by *HindIII*-*ApaI*. The arrow indicates the fragment CFP-54bp-YFP-C99, about 1.8 kb. Lane C, pcDNA3.0-CFP-54bp-YFP-C99 double-digested by *XbaI*-*ApaI*. The arrow indicates the fragment C99, about 300 bp. Lane D: pcDNA3.0-CFP-54bp-YFP-C99 double-digested by *XhoI*-*XbaI*. The arrow indicates the fragment YFP, about 720 bp.

the authenticity of the constructs. In agarose gel, four bands corresponding to 702 bp, 720 bp, 300 bp and 1.8 kb were observed as expected (Fig. 1). The sequencing results were identical to the corresponding GenBank sequences (details not shown).

3.2 FRET observation in transfected cells The scheme of FRET in the constructs is shown in Fig. 2. FRET occurred only in the cells expressing CFP-54bp-YFP (Fig. 3). A strong FRET signal was detected in the pcDNA3.0-CFP-54bp-YFP transfected cells at 12 h, 24 h, 48 h and 72 h post-transfection,

Fig. 2 Scheme of APP proteolysis and FRET occurrence. A: APP proteolysis. APP releases A β through the β -cleavage at β -site and the γ -cleavage at γ -site. The Swedish mutation is adjacent to the β -site. The APP717 mutation is near the γ -site. B: Illustration of FRET. FRET images are acquired at the emission wavelength of YFP (525 nm) when CFP is excited at 433 nm. FRET occurs before β cleavage: excitation of the CFP results in YFP emission. FRET disappears after β -cleavage, i.e. no YFP emission occurs when CFP is excited at 433 nm and only CFP images are acquired at its emission wavelength of 473 nm.

Fig. 3 Observation of FRET in CFP-54bp-YFP-transfected cells at 12 h, 24 h, 48 h, and 72 h after transfection. FRET strengthened gradually, then diminished long with the expression of CFP-54bp-YFP. Scale bar, 200 μ m.

Fig. 4 FRET was absent in CFP-54bp-YFP-C99-transfected cells at 12 h, 24 h, 48 h, and 72 h post-transfection. Scale bar, 200 μ m.

Fig. 5 A β was generated in CFP-54bp-YFP-C99-transfected cells. A: No A β and only cyan fluorescence in CFP-54bp transfected cells; B: YFP-A β in YFP-C99 transfected cells. YFP-C99 was cleaved by γ secretease to generate A β . (YFP-A β was also confirmed by immunocytochemistry, but the result was not shown); C: After β and γ cleavag, cyans fluorescence spread in the CFP-54bp-YFP-C99-transfected cells; D: YFP-A β was generated in the CFP-54bp-YFP-C99-transfected cells and similar to that in the YFP-C99 transfected cells (B). The cell shape was abnormal. Yellow fluorescent deposition accumulated in the cytoplasm and cell membrane. Scale bar, 200 μ m.

Fig. 6 Immunocytochemistry of A β . A: A β deposition was dyed as red fluorescence in cells. The nuclear was dyed blue. B: YFP-A β deposited in the cytoplasm and cell membrane. C: The distribution of red fluorescence (A) was coincident to that of yellow fluorescence (B) (YFP-A β). It proved that CFP-54bp-YFP-C99 was cleaved by β and γ secretase to generate A β . The nuclear and CFP presented as blue fluorescence (B, C). Scale bar, 100 μ m.

Fig. 7 Cell viability of transfection cells. Viability of cells in the CFP-54bp-YFP-C99 group decreased significantly compared with the control group and CFP-54bp-YFP group, indicating that the intracellular accumulation of A β produced damage to these cells. * $P < 0.05$ vs control group, # $P < 0.05$ vs CFP-54bp-YFP group.

indicating that the 54bp was not cleaved from the CFP-54bp-YFP. No FRET was observed in the cells transfected with plasmid pcDNA3.0-CFP-54bp-YFP-C99, indicating that the distance between CFP and YFP were farther than 100 Å (Fig. 4), i.e. the 54bp was probably cleaved from the fragment CFP-54bp-YFP-C99, thus CFP and YFP were separated from each other and FRET was disappeared. We also found that YFP-A β accumulated in cells (Fig. 5).

3.3 Detection of A β by immunocytochemistry A β was dyed with red and the nuclear was dyed with blue. Red fluorescence was identical to the distribution of yellow fluorescence, indicating that CFP-54bp-YFP-C99 could be cleaved not only by β secretase but also by γ secretase. The double cleavage produced accumulating A β in cells (Fig. 6).

3.4 Viability of transfected cells Accompanying the intracellular A β accumulation, the viability of cells decreased

significantly compared with the control group and CFP-54bp-YFP group ($P < 0.05$), suggesting that the intracellular A β accumulation had an adverse effect on cells (Fig. 7).

4 Discussion

The principle component of the senile plaques characteristic of AD is A β , which is produced from APP by two proteolytic events. The β -secretase cleaves APP at the N terminus (β site) and thus yields a membrane-associated APP fragment with a 99-aa terminus (C99), and a γ -secretase cleaves the second cleavage site in the transmembrane domain of C99 (γ site) to release the A β (about 39 aa to 42 aa)^[1]. The imbalance among the generation, aggregation, deposition and clearance of A β will lead to senile plaque production.

A number of APP mutations are linked to the pathogenesis of early-onset familial AD (FAD). For example,

the Swedish double mutation (K670N/M671L) is immediately adjacent to the β -secretase site and enhances the release of A β ^[2]. There are three mutations at the APP residue 717 (APP717 mutations including V717I, V717F and V717G) near the γ -secretase site and all of them increase the production of the more amyloidogenic 42-aa form of A β [A β (1-42)]^[3-6]. The biochemical mechanisms and cellular compartments involved in the A β generation and deposition have not been fully elucidated. The sequential β and γ cleavage of APP and A β generation have not been demonstrated in living neuronal cells either.

We constructed the recombinant plasmids pcDNA3.0-CFP-54bp-YFP and pcDNA3.0-CFP-54bp-YFP-C99 successfully. The fragment 54bp harbored Swedish mutation (β -site mutation) while the fragment C99 harbored the APP717 mutation (γ -site mutation). FRET occurred in the cells transfected with pcDNA3.0-CFP-54bp-YFP, but not in the cells transfected with pcDNA3.0-CFP-54bp-YFP-C99. The results indicate that the CFP-54bp-YFP-C99 could be cleaved by the β secretase but the CFP-54bp-YFP could not. Since the difference between CFP-54bp-YFP and CFP-54bp-YFP-C99 is only the fragment C99, it is presumable that C99 is significant for β cleavage, and it probably functions by guiding APP to right places for β proteolysis. If C99 is lost or C99's function is perturbed, the β cleavage of APP will be blocked. Our data raise the possibility that it may be advantageous to interfere with C99 domain to inhibit the β cleavage, which initiate the production of A β , though the accurate mechanism about the signal-peptide-like role of C99 need to be further explored.

A striking feature of our current studies is that A β deposits are widespread in cells. Our results strongly suggest that A β may be generated in cells and then accumulate intracellularly. There is evidence that A β accumulates intraneuronally in the cerebral cortex and hippocampus prior to A β plaque formation at the early stage of AD^[9]. Our data demonstrate that A β aggregation is very obvious within the cell prior to its accumulation outside of the cell.

Although it is known that A β is released from APP by β cleavage and γ cleavage, the proteolysis of APP and the generation of A β were not demonstrated in living cells before. To visualize the processing of APP, FRET was used by us to analyze the living neuronal cells which express mutant APP. The occurrence of FRET in the SH-SY5Y cells transfected with CFP-54bp-YFP indicated the close proximity of CFP and YFP, consistent with the ab-

sence of β cleavage within the fraction of 54 bp; while the absence of FRET in the cells transfected with CFP-54bp-YFP-C99 indicated that CFP and YFP were farther apart, consistent with the existence of β cleavage in the 54 bp. This cell-based FRET assay enable us to observe the process of APP proteolysis and the deposition of YFP-labeled A β in living cells.

The construction of pcDNA3.0-CFP-54bp-YFP-C99 and FRET assay developed in the present study could be used to further research the metabolism of APP. Furthermore, it also provides a direct and useful tool to investigate the pathogenesis of AD and to evaluate promising drug candidates in living neuronal cells.

Acknowledgements: This work was supported by the Key Project of Ministry of Education, China (No. 10420). We thank Dr. Todd Golde (Neurogenetics Laboratory, Mayo Clinic, Jacksonville, FL, USA) for the generous provision of the plasmid pcDNA3.0-APP.

References:

- [1] Farzan M, Schnitzler CE, Vasilieva N, Leung D, and Choe H. BACE2, a β -secretase homolog, leaves at the β site and within the amyloid- β region of the amyloid- β precursor protein. *Proc Natl Acad Sci USA* 2000, 97: 9712-9717.
- [2] Citron M, Oltersdorf T, Haass C, McConlogue L, Hung AY, Seubert P, *et al.* Mutation of the β -amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. *Nature* 1992, 360: 672-674.
- [3] Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, *et al.* Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 1991, 349: 704-706.
- [4] Chartier-Harlin MC, Crawford F, Houlden H, Warren A, Hughes D, Fidani L, *et al.* Early-onset Alzheimer's disease caused by mutations at codon 717 of the β -amyloid precursor protein gene. *Nature* 1991, 353: 844-846.
- [5] Murrell J, Farlow M, Ghetti B, Benson MD. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 1991, 254: 97-99.
- [6] Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L, Jr Eckman C, *et al.* An increased percentage of long amyloid beta protein secreted by familial amyloid β protein precursor (β APP717) mutants. *Science* 1994, 264: 1336-1340.
- [7] Xu Q, Brecht WJ, Weisgraber KH, Mahley RW, Huang Y. Apolipoprotein E4 domain interaction occurs in living neuronal cells as determined by fluorescence resonance energy transfer. *J Biol Chem* 2004, 279: 25511-25516.
- [8] Kinoshita A, Fukumoto H, Shah T, Whelan CM, Irizarry MC, Hyman BT. Demonstration by FRET of BACE interaction

with the amyloid precursor protein at the cell surface and in early endosomes. *J Cell Sci* 2003, 116: 3339-3346.

[9] Echeverria V, Cuelllo AC. Intracellular A-beta amyloid, a sign for worse things to come? *Mol Neurobiol* 2002, 26: 299-316.

多光子激光扫描成像技术对活细胞内淀粉样前体蛋白裂解和 β -淀粉样蛋白生成的观察

李晓晴¹, 张苏明¹, 杨华静¹, 张智红²

¹ 华中科技大学同济医学院附属同济医院神经内科, 武汉 430030

² 华中科技大学生物医学光子学教育部重点实验室, 武汉 430030

摘要: **目的** 在活细胞内探究淀粉样前体蛋白(amyloid precursor protein, APP)的裂解和 β -淀粉样蛋白(amyloid beta, A β)的生成机制。**方法** 利用PCR扩增CFP(编码蓝色荧光蛋白), YFP(编码黄色荧光蛋白)和C99(编码APP最后99个氨基酸)三片段。含有54个碱基(编码APP中间18个氨基酸)的片段54bp由生物公司合成。CFP, YFP, 54bp和C99四个片段连入载体质粒pcDNA3.0得到重组质粒pcDNA3.0-CFP-54bp-YFP和pcDNA3.0-CFP-54bp-YFP-C99。将这两个重组质粒分别瞬时转染SH-SY5Y细胞。利用多光子激光扫描显微镜观察融合基因的表达, 荧光共振能量转移(fluorescence resonance energy transfer, FRET)检测APP的 β 裂解。免疫细胞化学和多光子激光扫描显微镜观察A β 的生成。MTT检测转染细胞活性。**结果** (1)限制性内切酶双酶切消化和测序分析鉴定重组质粒序列完全正确。(2)转染细胞内可以观察到蓝色和黄色荧光。(3)在pcDNA3.0-CFP-54bp-YFP转染细胞中存在FRET现象; 在pcDNA3.0-CFP-54bp-YFP-C99转染细胞中观察不到FRET现象。(4)免疫细胞化学和多光子激光扫描显微镜观察到在pcDNA3.0-CFP-54bp-YFP-C99转染的细胞中有A β 的生成。(5)A β 的沉积广泛分布于细胞内。(6)随着A β 在细胞内的产生和聚集, 细胞的活性逐步下降。**结论** C99对于APP的 β 裂解非常重要。早期A β 首先在细胞内生成并在细胞内形成广泛沉积。细胞内聚集的A β 会影响细胞活性, 带来不利结果。

关键词: 淀粉样前体蛋白; β -淀粉样蛋白; β 裂解; 荧光共振能量转移