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A novel approach for Aβ$_{1-40}$ quantification using immuno-PCR

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ABSTRACT

Several lines of evidence suggest that aggregation of the amyloid β-peptide (Aβ) in the brain is a trigger of Alzheimer's disease (AD). Thus, quantification of Aβ in several different types of samples from brain is fundamental for understanding AD pathogenesis. For analysis of the low levels of Aβ present in microdissected neurons, a more sensitive system than the ELISAs used today would be helpful. Here, we report a novel immuno-PCR (IPCR) system in which the lowest quantitative level of Aβ$_{1-40}$ is 2 attomol/μL. We use the novel IPCR to quantify the intracellular Aβ$_{1-40}$ levels in pyramidal neurons microdissected from human brain. We show that the level of Aβ$_{1-40}$ is around 10 attomol/neuron, and thus, only 3 neurons are needed for analysis.

1. Introduction

In Alzheimer's disease (AD), the presence of senile plaques and neurofibrillary tangles in postmortem brain confirms the clinical diagnosis (Tiraboschi et al., 2004; Wenk, 2003). Senile plaques mainly consist of the amyloid β-peptide (Aβ), which is produced by β- and γ-secretase cleavage of the Aβ precursor protein (Selkoe, 1999; Sinha and Lieberburg, 1999). The pathogenesis of AD is not fully understood, but it has been proposed that aggregation of Aβ is a trigger of the disease (Lott and Head, 2005; Saito et al., 2011). Therefore, analysis of Aβ in human brain as well as in animal models and cell culture is technically critical in basic research. For some samples the sensitivity of the conventional ELISAs is insufficient, and hence, a more sensitive technique is required.

In 1992 a new ELISA technique, in which a conventional ELISA is combined with the signal amplification power of PCR, was reported (Sano et al., 1992). Here we report a novel and more sensitive immuno-PCR (IPCR) system for measurement of Aβ$_{1-40}$ and show that it can be used for quantification of minute amounts of Aβ in microdissected neurons.

2. Materials and methods

2.1. Dot blot for selection of a capturing antibody

G2-10 and 5C3 antibody were purchased from The Genetics Company, Inc. (Schlieren, Switzerland) and Calbiochem (Merck, Darmstadt, Germany), respectively. The specificity of the antibodies was tested against Aβ from human and rodent. Aβs were obtained from BACHEM (Budendorf, Switzerland) as a trifluoroacetic salt. All Aβ peptides were dissolved and incubated for 20 h in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP). After removal of HFIP by nitrogen flow, Aβ peptide stocks were prepared in dimethylsulfoxide at a concentration of 100 μM. After loading of Aβ peptides (1 pmol/spot, diluted with deionized water) on a nitrocellulose membrane, the membrane was blocked with Block Ace (DS Pharma Biomedical Co., Osaka, Japan) for 2 h at room temperature. The primary antibodies were used at a concentration of 1 μg/mL in 10% Block Ace with 0.1% Tween-20, and incubated at room temperature for one hour. A washing step was repeated 5 times with 20 mL of phosphate buffered saline containing 0.1% Tween-20. Horseradish peroxidase-linked goat-anti rabbit IgG (1/50,000 v/v, GE Healthcare UK Ltd., NA, UK) in 10% Block Ace with 0.1% Tween-20 was applied to the membranes for 1 hour at room temperature. Detection of Aβ was

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β-peptide; IPCR, immuno-PCR; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; TBS, Tris-buffered saline; TBST, TBS containing 0.05% Tween-20; Ct, threshold cycle; RIPA buffer, radioimmunoprecipitation assay buffer; CA1, cornu ammonis 1.

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achieved using SuperSignal® West Dura (Thermo Fisher Scientific, IL, USA).

2.2. DNA-antibody-conjugate for PCR detection

An overview of the IPCR system is shown in Fig. 1a. For detection, the 6E10 antibody (anti-$A\beta_{1-16}$ monoclonal antibody, COVANCE, NJ, USA) was covalently conjugated with the oligonucleotide, 90-mer part of multi-cloning site of pBluescriptSK(-) single-stranded DNA; 5′-acgacggccagtgagcgcgcgtaatacgactcactatagggcgaattgggtaccgggccc-cccctcgaggtcgacggtatcgataagctt-3′. The custom conjugation of the DNA with the 6E10 antibody, by hydrozone via the benzaldehyde-modified 5′ end of the DNA at neutral pH, was performed at SoluLink (San Diego, USA). The concentration of the conjugated antibody was determined by quantification of protein using BCA protein assay kit (Thermo Fisher Scientific).

2.3. Quantitative PCR reaction using DNA-antibody conjugate

As a quantitative PCR compatible 96 well plate, we adopted Top Yield Module Plate (Nunc, Thermo Fisher Scientific). We applied TaqMan PCR detection system (Life Technologies Corporation, CA, USA) in which the forward and the reverse primers were 5′-CAGTGAGCGCGCGTAATA and 5′-GCTTATCGATACCGTCGACCT, respectively. The VicTamra probe, 5′-TCACTATAGGGCGAATTGGG, was used for detection. The reaction mixture contained 15 μL of 2× TaqMan universal Master Mix, 2.7 μL of 10 μM forward and reverse primer, 0.75 μL of 10 μM VicTamra probe and 8.85 μL of DNase free water. First, we checked the PCR reaction using DNA-antibody conjugate (Fig. S2). The thermal cycling step on PRISM 7000 (Life Technologies Corporation) was as follows: initial activation of PCR enzymes at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 60 s. Data collection was performed at the extension stage.

2.4. IPCR assay

Thirty μL of G2-10 antibody (10 μg/mL) diluted in borate buffer (pH 9.5) was coated on a Top Yield Module Plate (Thermo Fisher Scientific) at 4 °C for 20 h with a tight-plastic seal. The wells were washed three times with 150 mM NaCl, 20 mM Tris–HCl, pH 7.5 (TBS) followed by a blocking step with 250 μL/well of SEA BLOCK (Thermo Fisher Scientific) or Protein-Free Blocker (Thermo Fisher Scientific) at 4 °C for 20 h. Prior to adding samples to wells, all wells were washed four times with 250 μL of TBS containing 0.05% Tween-20 (TBST).

We added 15 μL of DNA–antibody conjugate (10 ng/mL) and $A\beta_{1-40}$ samples prepared by a serial dilution to 15 μL of radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris–HCl, pH 8, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Thus, a final concentration of the conjugate was 5 ng/mL. The $A\beta_{1-40}$ and DNA-antibody conjugate were incubated at 4 °C for 22 h with a plastic seal. A washing step was repeated four times with 250 μL of (RIPA) buffer followed by three times with TBST and twice with TBS. PCR reaction mixture (30 μL) was applied to the wells, and the quantitative PCR reaction was performed as described above.

2.5. Neuronal test sample by laser capture microdissection

The human brain sample was obtained from the Huddinge Brain Bank at Karolinska Institutet Alzheimer Disease Research Center, and the study was approved by the local ethical committee at Karolinska Institutet. The preparation of CA1 pyramidal neurons from human hippocampal sections by a laser capture microdissection was performed as described previously (Aoki et al., 2008). In brief, frozen sections mounted on a polyethylene naphthalate membrane-covered glass slide (Carl Zeiss AG, Oberkochen, Germany) were Nissl-stained after ethanol fixation. Five hundred CA1 pyramidal neurons were microdissected into LPC-microfuge tube caps (PALM tube, Carl Zeiss AG), containing 20 μL of RIPA buffer with Roche complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, USA). The captured neurons were finally diluted to 750 μL in RIPA buffer and sonicated for 10 min.

2.6. Data analysis

The standard curve fitting was performed by the GraphPad PRISM4 software.

3. Results

3.1. Specificity of capturing antibodies

The 6E10 antibody which recognizes $A\beta_{1-16}$ was used as detection antibody. For capturing antibody, two monoclonal anti-$A\beta_{40}$ antibodies were tested. The specificity and titer of antibodies were assessed qualitatively by a dot blot using human $A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{1-43}$, human arctic (Glu → Gly at position 22) $A\beta_{1-40}$ and $A\beta_{1-42}$, and rodent $A\beta_{1-40}$ and $A\beta_{1-42}$. The specificity of the antibodies was acceptable, and the 5C3 antibody showed slightly lower titer on a dot blot (Fig. S1).

3.2. Quantitative PCR reaction

The PCR reaction was checked by plotting the Ct (Threshold Cycle) values versus the amount of conjugated antibody. The correlation coefficient of the standard curve was 0.999, indicating good correlation (Fig. S2). The slope was −3.31, close to the theoretical value −3.32.

3.3. Selection of a blocking reagent

The blocking reagent has to be selected carefully, since it has a critical influence on the performance of the ELISA (Sentandreu et al., 2007). In order to explore an adequate blocking reagent with a combination of capturing antibody, we checked signal and background levels in the IPCR system using three femtomol/well of $A\beta_{1-40}$. We compared Protein-Free Blocker and Sea Block, and found the former to give a relatively lower background level, i.e. higher Ct values (Fig. 1b). However, in samples with $A\beta_{1-40}$, higher signal levels (i.e. lower Ct values) were observed in Sea Block. In summary, the combination of G2-10 and Sea Block was found to be better in the IPCR assay since the higher signal ratio (with Aβ/without Aβ) allows a broader dynamic range of quantification.

3.4. Comparison of the IPCR and a conventional ELISA

Next, we compared the performance of the IPCR with a conventional $A\beta_{40}$ specific ELISA. The assay was performed according to the instruction manual, with the exception that we used our $A\beta_{1-40}$ stock and RIPA buffer for preparation of the series of $A\beta_{1-40}$ concentrations. The standard curve for the IPCR was calculated by a 4-parameter equation, $y = d + ((a-d)/(1+(x/c)\hat{}b))$, while quadratic equation fitting was used for the conventional ELISA. The standard curves showed a high coefficient of correlation in both assays (Fig. 1c and d, $n = 2$). The Ct values from each concentration of synthetic peptide were measured by the IPCR and back-calculated using the IPCR standard curve (Fig. 1c). The limit of quantification for $A\beta_{1-40}$ was 0.03 femtomol/well. On the other hand, the lowest

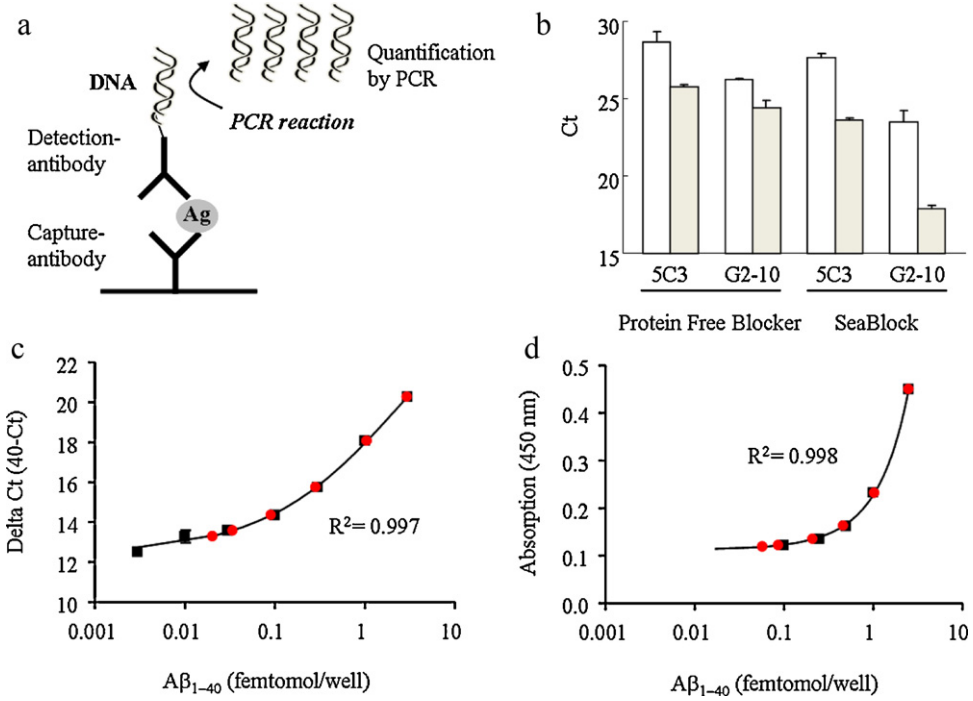

Fig. 1. Overview of the IPCR system and summary of experimental data in the IPCR. (a) Overview of the IPCR system, Ag: antigen. (b) Combinations of blocking reagents and antibodies. A combination of SeaBlock and G2-10 antibody demonstrated best performance on the IPCR system. Open and closed bars indicate Ct values in the absence or presence of 3 femtomol/well of $A\beta_{1-40}$, respectively. (c and d) Standard curves from the IPCR and the conventional ELISA. *X* axis was expressed by a logarithm scale. The signals from synthetic peptides on either the IPCR or the ELISA were measured and back-calculated using the obtained standard curves. Black square and red circles indicate observed and back-calculated values, respectively.

quantitative levels of $A\beta_{1-40}$ in the conventional ELISA was around 0.1 femtomol/well.

Next, we measured a neuronal sample obtained by laser capture microdissection, diluted one or three times, or spiked with 1 femtomol of $A\beta_{1-40}$. It was revealed that the neuronal sample contained 0.11 femtomol $A\beta_{1-40}$/15 μL/well ($n = 2$). In the three times diluted samples, the concentration was determined to be 0.044 femtomol/15 μL/well, indicating a good linearity of dilution. In the spiked sample, the concentration of $A\beta_{1-40}$ was determined to be 1.11 femtomol/well, which is in good agreement with the expected value (1.11 femtomol/well). Thus, we conclude that the present IPCR is more sensitive than the conventional ELISA, and that it is suitable for quantification of low concentrations of $A\beta_{1-40}$ in neuronal samples.

4. Discussion

Unlike a conventional ELISA, the IPCR system is based on a quantitative PCR for signal detection (Fig. 1a). We evaluated two monoclonal antibodies for capture of $A\beta_{40}$ by a dot blot assay and selected the most specific one. As detection antibody, we used the well documented 6E10 antibody. To avoid cross reactivity with contaminating nucleotides from mammalian materials, we conjugated a nucleotide sequence derived from pBluescriptSK(-) single-stranded DNA to the detection antibody.

For minimizing non-specific binding to the surface, we tested commonly used blockers such as bovine serum albumin, casein and nonfat dry milk. Most of them gave high back ground levels compared to Protein Free Blocker and SeaBlock (Fig. S3). We speculate that these natural blockers derived from mammals contain a trace level of 6E10 reactive species. Indeed, after immune adsorption by 6E10 the bovine serum albumin showed reduced background in the IPCR (Fig. S3). Between Protein Free Blocker and SeaBlock, we concluded that SeaBlock is more suitable for our system. The background level in Protein Free Blocker is likely to be low, suggesting more potent blocking efficacy than SeaBlock. However, Protein Free Blocker may cover an epitope for antibody recognition. Interestingly, 5C3 antibody showed similar performance in both these blockers, while it was evident that G2-10 antibody gave a higher signal to noise ratio in SeaBlock. Hence, we selected the combination of SeaBlock and G2-10 antibody.

The PCR was confirmed to be accurate and quantitative because the observed slope was consistent with a theoretical value, $1/\log_{10} 2$ (Fig. S2). Thus, the efficacy (E) in a PCR reaction was around 100% according to the equation, $E\,(\%) = (10^{-1/-3.31} - 1) \times 100$.

Both the IPCR system and the conventional ELISA showed a high correlation coefficient of the respective standard curve. By the IPCR system, the sensitivity of $A\beta_{1-40}$ measurement was improved by three times compared to the conventional ELISA. Moreover, it will be possible to lower the limit of quantification of $A\beta_{1-40}$ in the IPCR by increasing the sample volume, for instance to 100 μL/well. In that case, the lowest quantitative level would theoretically be 10 femtomol/well. Since a standard curve fitting in the IPCR was performed with a 4-parameter equation, in the lower range, i.e. under 0.03 femtomol/well, the standard curve slope versus the amount of $A\beta_{1-40}$ declined. This could explain why the lowest quantitative level was 0.03 femtomol/well despite a low standard deviation of the IPCR measurement in the whole range.

The method was tested on samples prepared from human hippocampus by laser capture microdissection. A neuronal sample was prepared in RIPA buffer at a concentration of 0.67 neurons/μL. The concentration of the original test sample (neuronal sample in Table 1) was determined to be 0.11 femtomol/15 μL/well. Thus, the neuronal concentration of RIPA soluble $A\beta_{1-40}$ is around 11 attomol/neuron. Next, we diluted the sample three times with RIPA buffer to investigate whether the IPCR showed a good linearity of dilution. The observed value was 0.044 femtomol/well, while the theoretical value calculated from an original neuronal sample was 0.037 attomol/well. These data suggest a good linearity of dilution and reveal that the lowest quantitative level is around

Table 1
Measurement of $A\beta_{1-40}$ and spike recovery test using neuronal samples.

Sample	Spiked (femtomol/well)	Measured (femtomol/well)	Theoretical (femtomol/well)	Recovery (%)
Neuronal sample	0	0.11 ± 0.01	–	–
3 times diluted neuronal sample	0	0.044 ± 0.016	0.037	119
Neuronal sample + 1 femtomol of $A\beta_{1-40}$	1.0	1.11 ± 0.09	1.11	100

The hippocampal sample prepared from CA1 region in RIPA buffer was used ($n = 2$).

30 attomol/well. The extrinsically spiked sample (a sample added by 1 femtomol of $A\beta_{1-40}$) was also measured with accuracy.

In conclusion, we report a novel approach for $A\beta_{1-40}$ analysis by quantitative PCR. The sensitivity was improved at least three times compared to the conventional ELISA, enabling quantification of $A\beta_{1-40}$ in cases where the sample amount is limited and the concentrations are low.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2012.01.015.

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