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Specificity and sensitivity of the Abeta oligomer ELISA

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ABSTRACT

Abeta soluble oligomers are believed to play a key role in the development of Alzheimer's disease (AD). An enzyme-linked immunosorbent assay (ELISA) commonly used to measure these proteins uses the same monoclonal antibody as both capture and reporter antibody. The objective of this study was to examine the specificity and sensitivity of this procedure, using monoclonal anti-Abeta antibody 6E10 as capture antibody and biotinylated 6E10 as reporter antibody. At comparable concentrations of Abeta soluble oligomers and low molecular weight (LMW) Abeta peptides, optical density (OD) values were four- to five-fold higher for the oligomer preparation than for the LMW Abeta. The LMW Abeta preparation, when evaluated by western blots of gels run under native conditions, showed only one band even after storage at 4 °C for more than two months, suggesting that the ELISA was detecting Abeta monomer as well as Abeta oligomers. Possible explanations for these results are that (1) the LMW Abeta preparation may contain Abeta oligomer species below the limit of detection of western blot, but still detectable by ELISA, or (2) some nonspecific binding of the LMW Abeta to the ELISA plate may have occurred, allowing its relevant epitope to remain available for binding by the reporter antibody. Because of the possibility that this ELISA may not be oligomer-specific, it seems prudent to suggest that it should be used in combination with other methods, rather than as the sole technique, for measuring Abeta oligomers in biological specimens.

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1. Introduction

Fibrillar amyloid-beta (Aβ) is the major protein within senile plaques in the Alzheimer's disease (AD) brain. The hypothesis that Aβ deposition may be the initiating event in AD pathology was first proposed in 1991 (Hardy and Allsop, 1991), but was difficult to reconcile with many studies indicating that plaque counts correlate poorly with the extent of dementia in AD (Terry et al., 1991; Arriagada et al., 1992; Bierer et al., 1995; Giannakopoulos et al., 2003). Intraneuronal accumulation of soluble Aβ is now thought to initiate the sequence of events leading to dementia (Wirths et al., 2004); in particular, soluble Aβ oligomers have been implicated in this process (reviewed by Lublin and Gandy, 2010). A number of techniques have been used to detect Aβ oligomers including the enzyme-linked immunosorbent assay (ELISA) (Howlett et al., 1997 and others; see below), immunocytochemical staining (Oddo et al., 2006), immunoblotting (Tomic et al., 2009), and western blot (Kayed et al., 2003). The ELISA is the most quantitative of these methods. The most commonly used ELISA for measuring soluble Aβ oligomers is a sandwich ELISA which employs the same monoclonal antibody as its capture antibody and, in its biotinylated form, as its reporter antibody. This procedure was first described by Howlett et al. (1997). A similar procedure, using different monoclonal antibodies, has been used by later investigators (Sian et al., 2000; El-Agnaf et al., 2000; LeVine, 2004; Xia et al., 2009). The procedure was stated in some of these studies to be specific for Aβ oligomers. The objective of the present study was to further examine the specificity and sensitivity of this procedure.

2. Materials and methods

2.1. Production of low molecular weight Aβ1–42 and soluble Aβ1–42 oligomers

Low molecular weight (LMW) Aβ was prepared as described previously, with slight modifications (Klaver et al., 2010). (In that study we referred to the preparation as Aβ monomer; here we have chosen to refer to it as LMW Aβ, in keeping with earlier studies which discussed the equilibrium between different soluble Aβ

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HFIP, hexafluoro-2-propanol; LMW, low molecular weight; OD, optical density; PBS, phosphate buffered saline; PBS–T, PBS with 0.05% Tween-20; SEM, standard error of the mean; TFA, trifluoroacetic acid.

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conformations [Bitan et al., 2001; Teplow, 2006].) Aβ1–42 (0.5 mg; AnaSpec, San Jose, CA) was disaggregated by resuspending in 0.25 ml trifluoroacetic acid (TFA, Sigma–Aldrich, Inc., St. Louis, MO) followed by hexafluoro-2-propanol (HFIP, Sigma–Aldrich). It was aliquoted into eppitubes (20 μl/tube), dried overnight (16–20 h) at room temperature in the fume hood, and stored at −20 °C. LMW Aβ was produced by resuspending the Aβ1–42 in HPLC-grade water adjusted to pH 3.0 with TFA (1 μl TFA per 10 ml HPLC H_2O). 0.6 ml TFA water was added to the Aβ-containing eppitube, and after thorough vortexing, this was put on ice in a separate tube. This procedure was repeated twice more on the same eppitube, yielding 1.8 ml of Aβ in TFA water. Tris base (21.8 mg) was added to bring the Tris concentration to 100 mM, and 3.8 μl of 12.1 N HCl was added to adjust the pH to 8.8. It was briefly centrifuged (11,752 × *g*, 5 min), passed through a 0.2 μm filter (GHP Acrodisc 13 mm syringe filter with 0.2 μm GHP membrane, Gelman Sciences, Ann Arbor, MI), and used immediately. The protein concentration, measured after filtration, was 6 μg/ml with the Bio-Rad protein assay.

Aβ oligomers were also produced as described previously (Klaver et al., 2010). 4.8 μl of 1% NH_4OH (supplied by AnaSpec) was added to an eppitube of disaggregated Aβ, and after brief vortexing, the tube sat for 1 min. The contents of the tube were then transferred sequentially to two more Aβ eppitubes, following this same procedure each time. It was then sonicated (water bath sonication, 4 min), followed by incubation for 1 h at room temperature. This yielded an Aβ concentration of 12.5 mg/ml. The preparation was then diluted in phosphate buffered saline (PBS; 0.01 M, pH 7.4, with 0.02% azide) to a final concentration of 58 μg/ml (per protein assay; data not shown). It was used immediately or stored at 4 °C for up to one week.

2.2. Evaluation of Aβ conformations

Western blots of LMW Aβ and Aβ oligomer preparations were performed under reducing/denaturing conditions as described in our previous study (Klaver et al., 2010), using 4–20% Tris-HCl Ready Gels (Bio-Rad Laboratories, Hercules, CA). Western blots of these Aβ preparations were also performed under native conditions. 23 μl of the Aβ monomer preparation (6 μg/ml; total amount added per lane, 0.138 μg) was mixed with an equal volume of Native Sample Buffer (Bio-Rad), then loaded into the appropriate lane. Standards from Sigma–Aldrich's Non-Denaturing Molecular Weight Kit (cat. # MWND500) were run in additional lanes. For both reducing/denaturing and native gels, the electrophoresed proteins were then transferred to Westran S PVDF membranes (Whatman International Ltd., Maidstone, UK) and western blot was performed, using mouse anti-Aβ monoclonal antibody 6E10 (Covance Research Laboratories, Berkeley, CA; 1:5000 dilution). Membranes were developed in SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL), and bands were detected on CL-XPosure film (Thermo Scientific).

The Thioflavin-T assay (LeVine, 1999) is the most frequently used procedure for monitoring fibril growth. It was used in the present study to determine if fibrils would develop in Aβ oligomer preparations during the course of the experiments. Thioflavin-T (Acros Organics, Fairlawn, NJ) was prepared as a 0.25 mM stock solution (pH 6) in 100 mM sodium phosphate and 100 mM sodium chloride. The solution was mixed at a 25:1 ratio with Aβ oligomer preparations, either freshly prepared or incubated overnight at 4 °C followed by 1 h at 37 °C. After vortexing, the mixture sat at room temperature for 30 min, then was mixed by trituration and aliquoted (100 μl per well) into eight wells of a black opaque 96-well culture plate (PerkinElmer, Waltham, MA). The plate was read at 37 °C with excitation at 450 nm and absorption at 485 nm on a Molecular Devices SpectraMAX Gemini EM microplate spectrofluorometer (Molecular Devices Corp., Sunnyvale, CA). Softmax Pro Version 5.0 (Molecular Devices) was used to evaluate the data.

2.3. Aβ oligomer ELISA

The sandwich ELISA to measure Aβ1–42 oligomers was performed as described by LeVine (2004) with slight modifications. Mouse monoclonal antibody 6E10 was diluted 1:400 in carbonate buffer (0.06 M, pH 9.8; 45.3 ml of 1 M $NaHCO_3$ + 18.2 ml of 1 M Na_2CO_3, brought to final volume of 1 L with dH_2O) and incubated on a 96-well Nunc Maxisorp plate (Nalge Nunc Bioproducts, South Bend, IN) overnight at 4 °C. The plate was washed three times with PBS (0.01 M, pH 7.2) with 0.05% Tween-20 (PBS–T); this wash was repeated after all incubation steps. Standard curves of the Aβ1–42 soluble oligomer and LMW Aβ monomer preparations were generated. The oligomer preparation was first diluted in PBS–T with 1% bovine serum albumin (hereafter, PBS–T–BSA) to 1720 ng/ml, then further diluted four-fold in the same buffer to 0.1 pg/ml. The LMW Aβ preparation was four-fold diluted from 1531 ng/ml to 0.4 pg/ml. Each dilution was placed in two wells (100 μl per well) and PBS–T–BSA was placed in three to six blank wells. In one experiment, unknowns consisting of high and low concentrations of Aβ1–42 soluble oligomers (high concentration 1.72 μg/ml, low concentration 6.7 ng/ml) and LMW Aβ (high concentration 6 μg/ml, low concentration 23.4 ng/ml) were placed randomly on the plate (12 wells/condition) to determine the ability of the ELISA to differentiate between these samples. The individual performing the ELISA was blinded to the conformations and concentrations of the samples. Incubation was continued overnight at 4 °C. The plate was treated with SuperBlock (SuperBlock Blocking Buffer in PBS, Thermo Scientific), applying it three times and removing it 1 min later, followed by three PBS–T washes. The plate was then incubated with biotinylated monoclonal antibody 6E10, diluted 1:1000 in PBS–T–BSA (1 h, 37 °C), followed by streptavidin–alkaline phosphatase (Zymed Laboratories, Invitrogen, Carlsbad, CA; 1:1000 in PBS–T; 1 h, 37 °C). Para-nitrophenol phosphate (Sigma; 5 mg in 40 ml of 1 M diethanolamine buffer, pH 9.8) was then added, and the plate was read at 405 nm with a V_{max} kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA) until the optical density (OD) of the oligomer standard curve reached 1.0. This experiment was performed on three separate occasions.

3. Results

3.1. Western blots and Thioflavin-T assays of Aβ preparations

Representative western blots are shown in Figs. 1 and 2. The freshly prepared LMW Aβ preparation produced one band, which migrated slightly below the lowest molecular weight standard, 7 kDa, in the reducing/denaturing western blot (Fig. 1, lane A). This band is likely to represent Aβ monomer (molecular weight 4.5 kDa). A western blot of the LMW Aβ preparation run under reducing/denaturing conditions after storage at 4 °C for 89 days showed this same band, plus a higher molecular weight band at the top of the gel, indicating that Aβ aggregates did eventually form in the LMW Aβ preparation after prolonged storage (Fig. 1, lane B). A western blot of a native gel with freshly prepared LWM Aβ contained one band which migrated below 14 kDa, the lowest native molecular weight standard; a faint protein smear was present just above this band, but no other bands were seen (Fig. 1, lane C). After storage of the LMW Aβ preparation at 4 °C for 74 days, still only this one band was observed (Fig. 1, lane D). After 96 days of storage at 4 °C, the only band seen was at the top of the gel, presumably corresponding to the high molecular weight band seen in the reducing/denaturing gel of this preparation after

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