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Inhibition of amyloid- β production by anti-amyloid precursor protein antibodies in primary mouse cortical neurones

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Abstract

Current therapies for Alzheimer's disease (AD) only treat the symptoms of the disease. We have previously developed a novel monoclonal antibody, 2B3, which binds to the β -secretase cleavage site in amyloid precursor protein (APP) and reduces the production of amyloid- β (A β) in human cell lines. To determine whether the antibody was likely to be effective in mouse models of amyloid pathology *in vivo*, we investigated whether 2B3 could also bind to APP in mouse primary cortical neurones.

Primary cortical neurones were produced from E15.5–17.5 C57Bl/6 wild-type and transgenic APP/V717I (London mutation) embryos. The percentage of the neuronal population was determined with immunocytochemistry. Cells were treated with 10 μ g/ml 2B3 or an irrelevant IgG for 48 hours and A β 40 levels determined by ELISA.

The population of cells was found to contain over 75% neurones and 2B3 bound effectively to these cells. No differences in A β 40 were detected between wild-type and transgenic cells. Importantly, 2B3 significantly inhibited the production of A β 40 by $75.15 \pm 1.37\%$ of the media control, while an irrelevant IgG only significantly reduced A β 40 levels by $23.35 \pm 5.55\%$ of the media control. The reduction in A β 40 produced by 2B3 was significantly greater than that caused by the IgG.

These data indicate that 2B3 binds to APP in mouse neurones and can inhibit A β 40 similarly to our previous findings. The antibody is probably therefore acting by steric hindrance of β -secretase and these data suggest that it will be effective in mice *in vivo* and could be an alternative potential therapy for AD.

Keywords

Alzheimer's disease; monoclonal antibodies; amyloid- β ; β -secretase cleavage site; amyloid precursor protein; primary mouse cortical neurones

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease for which there are currently only symptomatic treatments available in the U.K. [1]. There is therefore much interest in developing therapies which could treat the underlying cause of the disease. The predominant hypothesis to explain the onset of AD is the 'amyloid hypothesis' which suggests that aggregation of the small peptide, amyloid- β ($A\beta$) into toxic oligomers is an initiating event in the disease, leading to the formation of plaques, intracellular tau-containing tangles and neuronal loss [2-5]. Although the amyloid hypothesis has limitations, it does suggest that interfering with the production of $A\beta$ from its parent molecule, amyloid precursor protein (APP) should lead to successful therapies. $A\beta$ is made from APP by the sequential action of two enzymes, β -secretase, now identified as BACE1 [6-9], and the γ -secretase complex [6, 10-12]. We have developed a novel antibody, 2B3, which binds to the β -secretase cleavage site in APP and inhibits the production of $A\beta$ by steric hindrance [13]. Having demonstrated that 2B3 acts by inhibiting the activity of β -secretase, it is possible that it could be used as a novel therapeutic agent to treat AD. However, our previous work with 2B3 all involved the use of a human cell line and was performed *in vitro* [13]. In order to determine whether 2B3 is effective *in vivo* to reduce $A\beta$ production, we need to test it in transgenic mouse models of amyloid pathology. However, firstly we have to show whether 2B3 can inhibit $A\beta$ production in mouse neurones. Therefore the aim of this study was to investigate whether 2B3 can bind to APP in primary cortical mouse neurones from wild-type mice or transgenic mice expressing the London mutation in human APP (V717I) [14] and then reduce $A\beta$ levels. Adult animals with the London mutation mice have levels of human APP 2-5 times greater than endogenous APP and display increasing levels of $A\beta$ over the age of 12 months [14]. Our data clearly show that 2B3 significantly decreased the production of $A\beta_{40}$ in mouse cortical neurones thus supporting its use in transgenic models of amyloid pathology *in vivo*.

Methods

Materials and cell culture

All chemicals and reagents were purchased from Sigma-Aldrich, Poole, U.K., Life Technologies (Invitrogen), Paisley, U.K. or Fisher Scientific, Leicester, U.K. and all reactions were performed at room temperature unless otherwise specified.

Antibody production

Full details of the immunisation protocol, hybridoma development and antibody characterisation are detailed elsewhere [13,15]. 2B3 was raised to a 15-mer peptide spanning the β -secretase cleavage site on APP, EEISEVKMDAEFRHD. The antibody was concentrated from culture medium using Amicon Centriplus YM-100 filters (Millipore, Watford, U.K.) with a nominal molecular weight cut off of 100kDa and quantified by ELISA [13,15].

Primary cell culture

Animals were allowed free access to food and water and were housed at room temperature ($22\pm 2^{\circ}\text{C}$) with the lighting maintained on a 12h:12h light-dark cycle. This work complied with the guidelines for the care and use of laboratory animals according to the Animals (Scientific Procedures) Act 1986. The APP(V717I) London mutation [14] was maintained on the inbred C57Bl/6 background. Wild type C57Bl/6 female mice (Cathays Park Transgenic Production Unit, Cardiff University) were time-mated with heterozygous transgenic APP(V717I) males, a generous gift from Prof. Fred van Leuven (Katholieke Universiteit Leuven, Leuven, Belgium). The females were sacrificed at gestational day E15.5-17.5. Genotyping was carried out on DNA extracted from tail tips using standard methods and the following primers (Eurofins, MWG Operon, Ebersberg, Germany) were used to identify embryos with the APP(V717I) mutation: sense primer 5'-CCGATGGGTAGTGAAGCAATGGTT-3' and antisense primer 5'-TGTGCCAGCCAACAGAGAAAAC-3'.

Foetal cortical tissue from both hemispheres was dissected from transgenic V717I and wild type embryos into Hank's Buffered Salt Solution (HBSS) at 4°C , washed and collected by centrifugation. Cells were dissociated from the tissue by incubation with $1\mu\text{g/ml}$ trypsin (Worthington, Lakewood, U.S.A.) for 20 minutes at 37°C , the action of which was subsequently inactivated by the addition of $1\mu\text{g/ml}$ trypsin inhibitor and $50\mu\text{g/ml}$ DNAase (Worthington). Cells were spun down and triturated gently in expansion media (Dulbecco's modified Eagle's medium (DMEM) and HAM's F12 (1:1), supplemented with $1\times$ B27supplement (changed to $1\times$ N2 supplement after 1 week in culture), 20ng/ml fibroblast growth factor (FGF), 20ng/ml epidermal growth factor (EGF), 1 Unit/ml penicillin, $100\mu\text{g/ml}$ streptomycin, $1.25\mu\text{g/ml}$ Fungizone) and allowed to form neurospheres. Neurospheres were expanded for up to two weeks at which point they were dissociated and differentiated onto 30,000-70,000 kDa poly-L-lysine-coated ($20\mu\text{g/ml}$) coverslips in differentiation media (DMEM and HAM's F12 (1:1), supplemented with $1\times$ B27supplement, 1% foetal bovine serum and antibiotics as above) at a concentration of 125,000 cells per well of a 24-well plate. All centrifugation steps were performed at 300g, all enzymes were diluted in HBSS and all tissue from different embryos was maintained separately. Neurones were used 24 hours after differentiation.

Immunocytochemistry

Differentiated neurones on coverslips were fixed and processed for immunocytochemistry as previously described [15]. Cells were incubated with antibodies to neurone-specific enolase (NSE, $10\mu\text{g/ml}$, Abcam), glial fibrillary acidic protein (GFAP, $1\mu\text{g/ml}$, Abcam) or 2B3 ($10\mu\text{g/ml}$) in blocking solution (0.1M phosphate-buffered saline (PBS), 3% serum from the species used to raise the secondary antibody, 1% bovine serum albumin) overnight at 4°C . Antibody labelling was detected using appropriate secondary antibodies conjugated to biotin (1:500, Vector Laboratories) followed by avidin-FITC (1:600, Vector Laboratories). Cover slips were mounted on to glass slides using VECTASHIELD® HardSet™ Mounting Medium (Vector Laboratories) with DAPI (1:4) and visualized using a Leica DMIRE2 microscope and Openlab 4.04 software. All images were taken with a $63\times$ objective lens.

Effects of 2B3 on levels of A β 40

Differentiated neurones were incubated with control media, 2B3 or an irrelevant control mouse IgG (Sigma-Aldrich) (both at 10 μ g/ml) for 48 hours at 37°C. For analysis of A β 40, media was subjected to immunoprecipitation and ELISA as described previously [13,15]. Briefly, the ELISA employed the N-terminal A β antibody 6E10 (5 μ g/ml), as the capture antibody and affinity-purified BAM401AP (0.45 μ g/ml Autogen Bioclear, Calne, U.K.), specific to the C-terminus of human A β 40, as the detection antibody. Data were normalised to total cell protein concentration as determined by BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL, U.S.A.).

Statistical analysis

Data generated in ELISA assays were quantified by comparing data to standard curves included on each plate, using Graphpad Prism® 4. Results were first normalised to total protein concentration, where relevant, expressed as % of media control values and then either compared to the 'hypothetical' control value of 100% or using unpaired Student's t-tests, both at the two-tailed significance level.

Results

Cells from each embryo were maintained and processed separately for all experiments. No significant differences were seen in A β 40 levels between cells from transgenic (2.33 $\pm$ 0.58ng/mg protein, n=3) and wild type animals (2.39 $\pm$ 0.33ng/mg protein, n=4) and APP levels were also similar as seen with immunocytochemical labeling for 2B3 and two commercial anti-APP antibodies detecting the N and C termini of APP, respectively (data not shown). Data for transgenic and wild-type embryos were therefore pooled for the A β 40 ELISAs.

Immunocytochemical staining using NSE as a neurone-specific marker confirmed the presence of a population of differentiated neurones estimated to be over 75% of the cells in the cultures from all embryos (Figure 1A). A few cells were stained with GFAP indicating they were glial cells (Figure 1B). 2B3 labelled many cells in cultures from both wild-type and transgenic embryos (Figure 1C). Negative controls were performed for all antibodies by exclusion of the primary antibody and showed no specific staining (Figure 1D for 2B3, data not shown for NSE or GFAP). Images for cells from wild-type and transgenic embryos were similar and therefore only wild-type cells are shown in Figure 1.

Administration of 2B3 at 10 μ g/ml, a concentration we have previously shown to reduce the production of A β 40 in cells in culture [13], to primary cortical neurones resulted in a significant 75.15 $\pm$ 1.37% (p<0.0001) reduction from media control. Treatment with the irrelevant IgG produced a much smaller, significant reduction of 23.35 $\pm$ 5.55% (p<0.006) in A β 40 levels compared to media-treated cells (Figure 2). Importantly, the reduction in A β 40 produced by 2B3 was significantly different to that caused by the irrelevant IgG (p<0.0001). Attempts were made to measure A β 42 in the neurones as we have previously shown that 2B3 also affects A β 42 production [13], but levels were below the detection limit of the ELISA.

Discussion

We have clearly demonstrated that our anti- β -secretase cleavage site antibody, 2B3, can bind to primary mouse cortical neurones and inhibit the production of A β 40. Thus our antibody should bind to APP in mouse brains *in vivo* and inhibit the production of A β 40.

We successfully obtained cultures of cortical mouse cells consisting of over 75% neurones. Therefore, it is highly likely that the results we have obtained are predominantly due to the binding of 2B3 to APP in neurones rather than glial cells. However, as glial cells also produce A β (16), 2B3 could also have a beneficial effect to reduce A β made by these cells. Furthermore, we found here that 2B3 can bind to these mouse neurones showing that the antibody recognises murine APP as well as human APP in cell lines [13]. We saw no differences in A β 40 or APP levels comparing the wild-type and transgenic embryos. This is due to lack of activation of the transgene, the Thy1 promoter used in the construct remains inactive until after birth [17]. Therefore we were able to pool the data from the A β 40 ELISAs for the wild-type and transgenic embryos.

We obtained a large 75% reduction in A β 40 production that was similar to the 63% reduction we have previously described for 2B3 in immortalized human MOG-G-UVW astrocytoma cells [13]. In our other studies with 2B3, we saw no effect of the antibody on non-amyloidogenic cleavage by α -secretase [13] so we think it highly unlikely that this pathway would be affected in the cortical neurones. We therefore propose that 2B3 is likely to be working by the same mechanism of steric hindrance of β -secretase activity that we described previously for the human cells [13]. The small effect of the irrelevant control IgG is likely to be due to the inclusion of this large protein in the incubation media of the cells and provides a control for this possible effect of 2B3. Importantly, the much larger effect of 2B3, and the clear difference between the 2B3 and IgG-treated groups, demonstrates that it is not simply the presence of a monoclonal murine antibody that produces the reduction in A β 40 levels reported here. Clearly, it is essential to have an antibody that can bind to the β secretase cleavage site [13].

The data we have described here strongly suggest that 2B3 will bind to mouse neurones in the brain and may reduce A β production *in vivo*. Since 2B3 binds to endogenous mouse APP, it could be used to investigate the role of APP in normal brain function. Importantly, our results will now allow us to test the efficacy of 2B3 to both reduce A β levels and to improve cognitive performance in transgenic mouse models of amyloid pathology. Such studies are critical to determine whether this anti- β -secretase cleavage site antibody could be a potential novel therapeutic agent for use in AD patients.

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Figure 1.

Representative examples of immunocytochemistry for NSE, GFAP and 2B3 in mouse primary cortical cultures (A-D). (A) Specific labelling of a large population of wild-type neurones with an anti-NSE antibody (10 μ g/ml) detected using a biotinylated anti-rabbit antibody (1:500) and Avidin-FITC (1:600); (B) specific labelling of a small population of wild-type glial cells with an anti-GFAP antibody (1 μ g/ml) detected as above; (C) specific labelling of wild-type cells with 2B3 (10 μ g/ml) detected using a biotinylated anti-mouse antibody (1:500) and Avidin-FITC (1:600); (D) negative control for 2B3 showing low levels of non-specific staining in wild-type cells incubated in the absence of the primary antibody and detected as above. Similar results were obtained with negative controls for NSE and GFAP (data not shown). Coverslips were mounted with VECTASHIELD mounting medium with DAPI to counter-stain the nuclei. n = 5-7. Scale bar = 25 μ m.

Figure 2.

Levels of extracellular Aβ40 from culture media of primary cortical neurones from wild-type and transgenic APP V717I mouse embryos after incubation with 2B3 or an irrelevant mouse IgG at 10μg/ml for 48 hours. Data are expressed as mean (± SEM) % of media control Aβ levels as detected in a sandwich ELISA and corrected for total cell protein concentration. Data were pooled for wild-type and transgenic embryos. 2B3 significantly reduced Aβ40 from media control and the irrelevant IgG. The irrelevant IgG produced a much smaller, significant decrease in Aβ levels from media control. **p<0.01, ***p<0.0001, significantly different from media control (100%); ###p<0.0001, 2B3 significantly different from IgG with unpaired two-tailed Student's t-tests. n = 7.