Characterization of Amino Acid Residues Integral to Neuronal Binding of Amyloid Beta Protein in Alzheimer's Disease

by

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I. BACKGROUND

I spent the summers of 2009 and 2010 working at the Mayo Clinic in Rochester, Minnesota, as part of their Graduate School's Summer Undergraduate Research Fellowship (SURF) program. The SURF program provides the opportunity for undergraduate students from universities across the country to work in one of the many labs on the Mayo Clinic campus. In addition to working on a research project, SURF students present their results to their department at the end of the summer in the form of an oral presentation and a poster session, and are able to attend seminars almost daily during their time at Mayo. I spent both summers working in a laboratory in the Molecular Neuroscience Department under the direction of Joseph Poduslo, Ph.D. The work was directed towards understanding the mechanism of Alzheimer's Disease (AD) and developing an immunotherapeutic treatment. This report summarizes the work I completed towards these objectives.

II. INTRODUCTION

Alzheimer's Disease (AD) is a progressive and fatal neurodegenerative condition. AD is the most common form of senile dementia, accounting for roughly 60% of all dementia cases, and is the fourth leading cause of death in developed nations. Today, over 5 million Americans are living with AD and there are at least 26.6 million people afflicted worldwide. Common symptoms of Alzheimer's Disease are memory loss, a decline in physical coordination, and personality changes.

AD is characterized pathologically by the presence of extracellular amyloid plaques, neurofibrillary tangles, cerebrovascular amyloid deposits, and intraneuronal accumulation of amyloid-beta peptides. These amyloid-beta peptides (Aβ) are produced by the cleavage of amyloid precursor protein, an integral membrane protein. When amyloid precursor protein (APP) is cleaved by both beta- and gamma-secretases, the neurotoxic peptide fragments amyloid-beta 40 (Aβ40) and amyloid-beta 42 (Aβ42) are formed, as in Figure 1 (page 3).

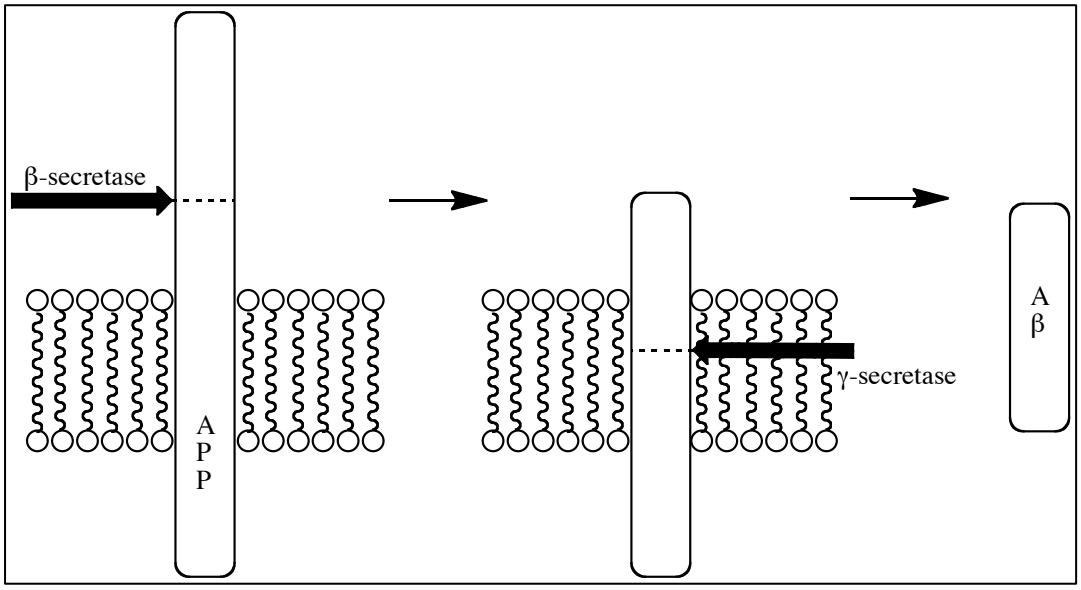

Figure 1. Processing of amyloid precursor protein (APP).

These peptides constitute extraneuronal plaques and deposits, and it is believed that the increased production and intraneuronal accumulation of these peptides leads to neurodegeneration and AD. Additionally, it has been suggested that soluble oligomers of Aβ, rather than amyloid fibrils or monomers, initiate the cognitive dysfunction observed in AD. My studies were performed using Aβ40, though the striking similarity between the two peptides (Aβ42 has two additional amino acids) suggests that the two behave similarly *in vivo*.

Previous research performed in the lab suggested that three amino acids present within both Aβ40 and Aβ42 are integral to the neuronal binding and internalization of these peptides. These amino acids are the histidine residues at positions 13 and 14, as well as the glycine residue at position 33. For the complete amino acid sequence of Aβ40, see Figure 2 (page 4).

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV

Figure 2. Amino acid sequence of Aβ40, with residues of interest in red.

The goal of my project was to block the neuronal binding and internalization of Aβ40. I attempted to do this using a human monoclonal antibody that binds to positions 2011 on Aβ (IgG-4.1). We hypothesized that IgG-4.1 would function as a blocking antibody to Aβ40 by sterically hindering the histidine residues at positions 13 and 14. This steric hindrance of key amino acids should interfere with the neuronal interaction of Aβ40, thereby prevents its binding and internalization.

My research also examined the roles that histidine-13, histidine-14, and glycine-33 play in the neuronal binding and internalization of Aβ40. I did this by studying not only native Aβ40, but also substituted derivatives of the peptide. I examined Aβ40 with the histidine residues at positions 13 and 14 substituted to glycine (Aβ40 H13,14G), and Aβ40 with an additional substitution of the glycine residue at position 33 to alanine (Aβ40 H13,14G; G33A. My hypothesis was that Aβ40 H13,14G and Aβ40 H13,14G; G33A would both have lowered levels of binding and internalization as compared to Aβ40.

III. EXPERIMENTAL METHODS

A cell line derived from the pheochromocytoma of the rat adrenal medulla called "PC-12" was used as a model cell system for human neurons. These cells stop proliferating and differentiate when treated with a protein known as nerve growth factor (NGF). When fully differentiated (after six days of treatment with NGF), PC-12 cells have grown dendrite-like extensions and wholly resemble neurons in both function and appearance.

PC-12 cells were maintained in a Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% horse serum, 10% newborn calf serum, and 1% penicillin/streptomycin. PC-12 cells were plated on six-well plates that had been treated with poly-L-Lysine. This poly-L-Lysine was applied to wells at a concentration of 0.1 mg/mL and was allowed to incubate in the wells overnight at 37°C. After the overnight incubation, the poly-L-Lysine was removed from the wells. The plates were rinsed twice with Dulbecco's Phosphate Buffered Saline without calcium and magnesium and allowed to dry for a minimum of two hours. The PC-12 cells were then plated at a cell density of 25,000 cells per well in supplemented DMEM containing 100 ng/mL Nerve Growth Factor (NGF). The PC-12 cells were then allowed to differentiate for six days. The supplemented DMEM media containing NGF was changed on the cells every two days during differentiation.

In order to allow for determination of the amount of peptide associated with the cell samples at the end of an experiment, peptides were synthesized with the fluorochrome fluorescein isothiocyanate (FITC). This fluorescent dye allowed for the determination of the geometric

mean fluorescence of the cells via flow cytometry, since flow cytometry allows for measurement of the fluorescence emission of cells. In a flow cytometer, the cell suspension is first focused into a single cell wide laminar flow column. This fluid column is then passed through a laser beam, which hits the cells one at a time as the column passes through the beam. When cells that had taken up FITC-labeled peptide pass through the laser beam, they emitted fluorescence that was detected and recorded by the flow cytometer. The geometric mean fluorescence of the cell samples was determined by the flow cytometer in this way, and was directly proportional to the amount of fluorescent-labeled peptide (F-Aβ40, F-Aβ40 H13,14G, or F-Aβ40 H13,14G; G33A) associated with the cells.

Differentiated PC-12 cells were treated with various experimental "plating mixes" and allowed to incubate at 37°C for one hour. These plating mixes contained one of the four different fluorescent-labeled peptides (5 μM peptide), or one of the fluorescent-labeled peptides (2.5 μM peptide) that had been pre-incubated with IgG-4.1 at a 1:1 ratio. A control plating mix containing only Hank's Balanced Salt Solution (with calcium and magnesium + 10 mM HEPES, pH 7.4) was used to measure the amount of background fluorescence, and a plating mix containing human transferrin conjugated to AlexaFluor633 (20 mM transferrin), a marker for endocytosis, was used to ensure healthy, viable cells.

After the one hour incubation time, the plating mixes were pulled off and the cells were removed from the wells using either a non-enzymatic Cell Dissociation Buffer (1X) or trypsin (0.0125%). The non-enzymatic treatment allowed for removal of the cells from the wells without cleaving off any peptide bound to the exterior of the cells, whereas trypsin

cleaved any peptide that was associated with the exterior of the cell, thereby leaving the cells with just the internalized peptide. The use of these two different treatments allowed for the differentiation between the total amount of peptide associated with the cells (as displayed by the geometric mean fluorescence of Cell Dissociation Buffer-treated cells) and the amount of peptide that had been internalized by the cells (as shown by the geometric mean fluorescence of trypsin-treated cells). After the cells were removed from the wells, they were fixed in 1% paraformaldehyde and analyzed on a FACSCalibur flow cytometer. This experimental set-up is summarized in Figure 3.

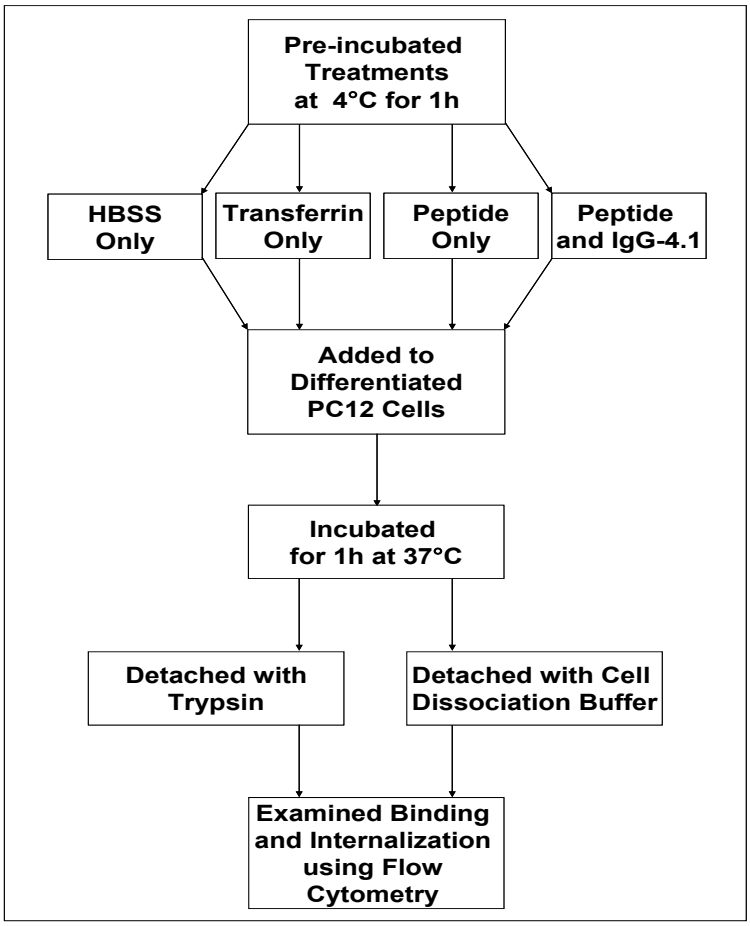

Figure 3. Flowchart providing a summary of the experimental procedure.

IV. RESULTS

The geometric mean fluorescence (GMF) values obtained from flow cytometry analysis of each sample were directly proportional to the amount of associated peptide, which allowed for GMF values to be used to quantify peptide binding and internalization. The obtained GMF values for three trials of the experiment are presented in Appendix A (page 13). The results of the experiments analyzing the effects of the various amino acid substitutions as well as the presence of antibody are summarized in the form of charts describing GMF for each experimental treatment in the following Figure 4 and Figure 5 (page 9). Figure 4 illustrates the GMF values for cells treated with cell dissociation buffer, representing the amount of total associated protein, while Figure 5 gives the GMF values for cells treated with trypsin, which represents the amount of internalized peptide. The cells treated with media only ("Cells Only") and with Transferrin were controls.

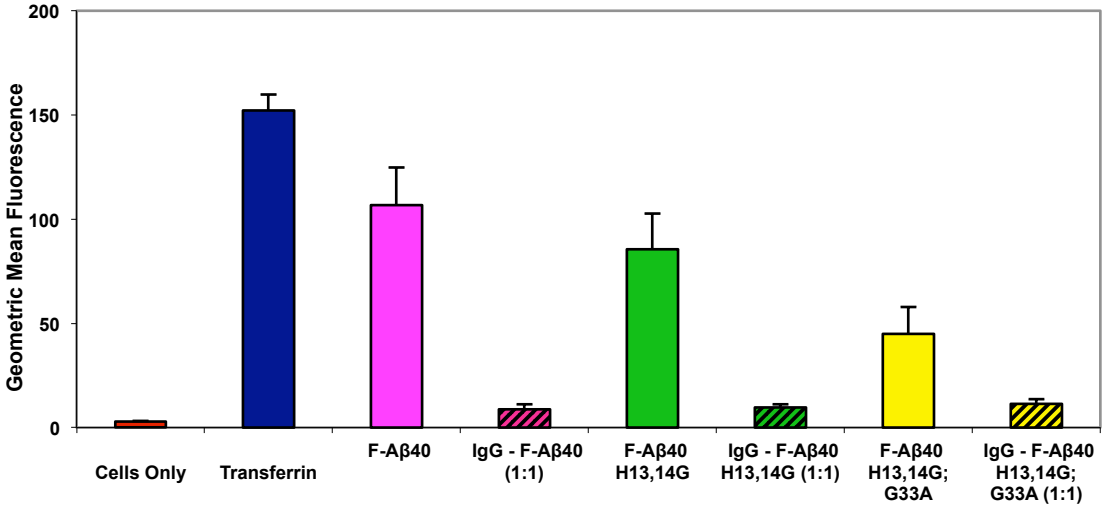

Figure 4. Geometric mean fluorescence of cells treated with cell dissociation buffer.

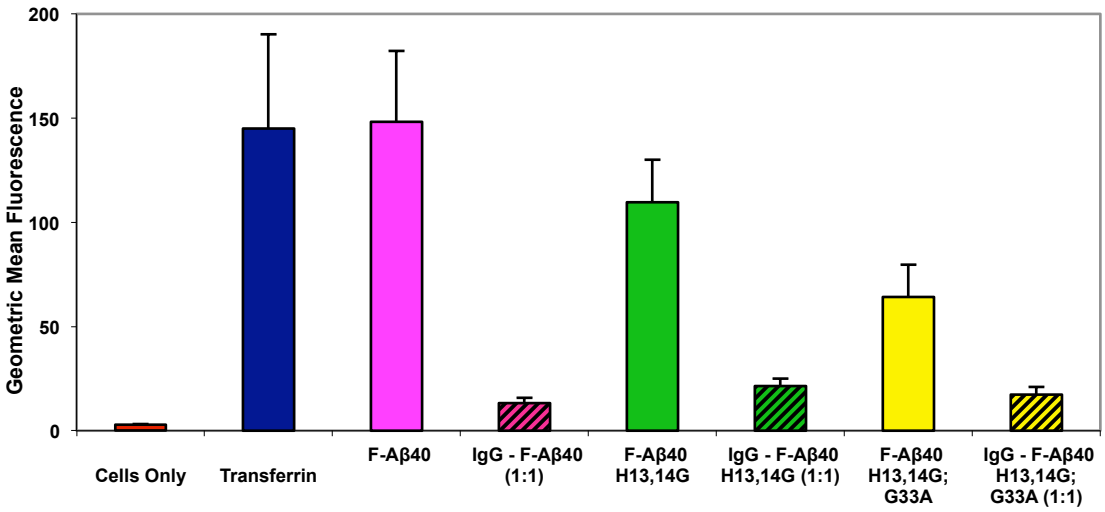

Figure 5. Geometric mean fluorescence of cells treated with trypsin.

The low GMF values for cells treated only with media suggest that the media used in the experiments did not interfere with the flow cytometry analysis. The high GMF values for cells treated with Transferrin reveals that the cells were healthy and were actively performing endocytosis. The Transferrin levels were similar between cells treated with trypsin and with cell dissociation buffer, which is as expected since Transferrin should be internalized via endocytosis and not remain bound to the membrane. By comparing Figure 4 and Figure 5, it can be observed that all GMF values of cells treated with trypsin are only slightly lower than those treated with cell dissociation buffer.

The GMF values for cells treated with substituted F-Aβ40 were lower than those for cells treated with wild type F-Aβ40. Each additional substitution further reduced the GMF value. Additionally, cells that were incubated with plating mixes containing a peptide that had been

pre-incubated with IgG-4.1 exhibited a significant decrease in GMF as compared to cells treated with peptide alone.

V. DISCUSSION and CONCLUSION

Several important conclusions can be drawn from the results presented in Figures 4 and 5. First, since the GMF values were very similar between cells treated with trypsin and cells treated with cell dissociation buffer, it is clear that the majority of F-Aβ40 associated with the cells was internalized by them, instead of remaining bound to the membrane. Secondly, the GMF values decreased with each additional amino acid substitution, confirming previous work that the histidine residues at 13 and 14 and the glycine residue at 33 are indeed important in Aβ40 being internalized and accumulated. Finally, and most significantly, cells that were incubated with plating mixes containing a peptide that had been pre-treated with IgG-4.1 exhibited a significant decrease in GMF as compared to cells treated with just the peptide. Clearly, the presence of IgG-4.1 blocked the internalization of F-Aβ40 as well as its substituted derivatives.

These results obtained from PC12 cells can be extended to the neuronal binding and internalization of F-Aβ40. Internalized peptide was found to be responsible for a large proportion of the total amount of peptide associated with the neurons, and very little of it remains bound to the membrane. Amino acid substitutions of F-Aβ40 decreased the levels of binding and internalization of F-Aβ40, confirming the importance of the histidine residues at positions 13 and 14 and the glycine residue at 33 in neuronal binding and internalization. Most importantly, pre-treating F-Aβ40 and its derivatives with IgG4.1 decreased their neuronal binding and internalization. This finding suggests that immunotherapy may play a

role in reducing the intraneuronal accumulation of Aβ40, thus preventing the initiation of the neurodegeneration associated with Alzheimer's Disease.

VI. APPENDIX A

Table 1: Geometric Mean Fluorescence Values for Three Experiment Trials

Trypsin	**22-Jul-07**	**27-Jul-07**	**28-Jul-07**	**Average**	**Standard Deviation**	**Standard Error**
Cells Only	2.2	3.1	3.5	3.0	0.7	0.39
Transferrin Only	151.1	139.6	165.8	152.2	13.1	7.57
F-AB40 Only	78.9	100.6	140.8	106.7	31.4	18.15
F-AB40/IgG-4.1	4.7	13.4	8.1	8.8	4.4	2.54
F-AB40 H13,14G Only	56.7	84.2	115.8	85.6	29.6	17.09
F-AB40 H13,14G/IgG-4.1	6.7	10.6	11.7	9.7	2.6	1.51
F-AB40 H13,14G; G33A Only	31.9	32.5	70.5	45.0	22.1	12.76
F-AB40 H13,14G; G33A/IgG-4.1	8.0	10.1	15.9	11.4	4.1	2.37
Cell Dissociation Buffer						
Cells Only	2.6	2.7	3.6	3.0	0.6	0.32
Transferrin Only	121.9	80.7	232.4	145.0	78.4	45.33
F-AB40 Only	135.5	96.9	212.3	148.2	58.8	33.96
F-AB40/IgG-4.1	9.5	11.9	18.3	13.2	4.5	2.63
F-AB40 H13,14G Only	104.2	77.6	147.1	109.6	35.1	20.28
F-AB40 H13,14G/IgG-4.1	15.7	20.8	27.9	21.4	6.1	3.55
F-AB40 H13,14G; G33A Only	54.5	43.6	94.4	64.2	26.7	15.45
F-AB40 H13,14G; G33A/IgG-4.1	10.0	22.8	19.2	17.3	6.6	3.80

VII. REFERENCES

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