Detecting Oligomeric Beta-Amyloid for the Diagnosis of Alzheimer's Disease

by

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ABSTRACT

Alzheimer's disease (AD) is the leading neurodegenerative disease, affecting roughly 8% of people 65 years of age or older. There exists an imperative need to develop a non-invasive test for the earlier detection of AD. The use of biomarkers is a promising option that examines the toxic mechanisms and metabolic pathways that cause Alzheimer's disease, eventually leading to an early diagnostic method. This thesis presents the use of oligomeric beta-amyloid as a biomarker to detect Alzheimer's disease via a specialized enzyme-linked protein assay. Specifically, this paper details the optimization and development of a novel phage capture enzyme-linked immunosorbent assay (ELISA) that can detect the relative quantity of beta-amyloid oligomers in samples from a mouse model of AD.

The objective of this thesis was to optimize a phage capture ELISA using the A4 single-chain variable fragment (scFv) to quantify the amount of beta-amyloid oligomers in various mice samples. A4 selectively recognizes a toxic oligomeric form of beta-amyloid. The level of A4-reactive oligomeric beta-amyloid was measured in triplicate in homogenized mouse brain tissue samples from eight transgenic (TG) and eight nontransgenic (NTG) animals aged five, nine, and thirteen months. There was a significant difference ($p < 0.0005$) between the five month TG and NTG mice. A decrease in beta-amyloid levels with the aging of the TG mice suggested that the beta-amyloid oligomers may be aggregating to form beta-amyloid fibrils. Conversely, the quantity of beta-amyloid increased with the aging of the NTG mice. This indicated that beta-amyloid oligomers may develop with normal aging.

DEDICATION

This thesis is dedicated to my loving parents, Jane and Joseph, and to my wonderful older sister, Morgan. Thank you for tolerating my unavailability during these past five years, and for your unwavering support. I will always treasure the rare weekends when the stars aligned, and I had an available Saturday to spend with my family: playing Taboo, being cropped out of photos of the dog, and leaving with Tupperwares full of home-cooked goodies. I cannot wait to begin this new chapter of my life with you, and I look forward to many more Saturday evenings of board games, laughter, and pie.

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INTRODUCTION

1.1 Disease Stages

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease which affects more than 5 million United States residents, a total that is projected to increase to 13 million by 2050 (Bateman et al., 2012). Mild-Cognitive Impairment (MCI) describes initial, nondisabling cognitive disorders, where patients diagnosed with early stages of Alzheimer's dementia are typically characterized by MCI (Petersen et al., 2001; Winblad et al., 2004). The characterization of MCI has been principally determined from clinical and neuropsychology studies, where its classification is continually updated (Cedazo-Minguez and Winblad, 2010). The brain damage present in those with MCI is less advanced and thought to be more treatable than that of Alzheimer's patients. For this reason, there are multiple nationwide, multidisciplinary studies to focus on individuals with MCI (Sperling et al., 2011). The cognitive and behavioral deterioration associated with Alzheimer's disease is divided into four distinct stages: 1) pre-dementia, 2) mild, 3) moderate, and 4) severe (Aisen, 2010; Kawas, 2003; Wilcock et al., 2000; Ishii, 1997; Förstl and Kurz, 1999).

1.1.1 Pre-Dementia

The initial symptoms of AD which signify pre-dementia include memory loss, decreased or poor judgment, and changes in mood and personality (10 Early Signs and Symptoms of Alzheimer's, 2009). These signs are frequently misinterpreted and credited to aging or stress (Waldemar et al., 2007). Of the indicators of pre-dementia, the most profound symptom is memory loss (Bäckman et al., 2004), where there are impairments in both the memory of experiences and specific events (episodic memory) and the memory of facts, meanings, and knowledge about the external world (semantic memory) (Episodic & Semantic Memory, 2010). Beginning in pre-dementia, apathy is the most significant neuropsychiatric symptom which continues throughout the course of the disease (Landes et al., 2001).

1.1.2 Mild

Mild Alzheimer's disease is marked by the continual decline in cognition and function which generally leads to a definitive diagnosis. Memory function continues to decline in this stage, where newer facts and memories are often forgotten. The episodic, semantic, and implicit memory, the memory in which previous experiences aid in the performance of a task (e.g., tying one's shoes) are mostly unaffected in the earlier stages of AD (Carlesimo and Oscar-Berman, 1992; Jelicic et al., 1995). While an individual with mild Alzheimer's disease does experience a diminishing vocabulary and word fluency, one can still sufficiently communicate basic ideas (Förstl and Kurz, 1999; Taler and Phillips, 2008; Frank, 1994). Supervision may be required with more cognitively challenging tasks, but those with early AD can continue to perform many activities independently (Förstl and Kurz, 1999). The loss of movement coordination and motor planning may be observed when one's fine motor skills are used (e.g., drawing or folding clothing); however, these impairments are often undiagnosed (Förstl and Kurz, 1999).

1.1.3 Moderate

After the clinical diagnosis of Alzheimer's, life expectancy is reduced by 33.3% (Heymen et al., 1987). With moderate Alzheimer's, disease advancement prevents independence; afflicted individuals cannot accomplish everyday tasks, such as using household appliances, dressing, and eating (Förstl and Kurz, 1999). Language difficulties become apparent as the frequency of incorrect word substitutions and identification increase (Förstl and Kurz, 1999). Spatial disorientation increases (Haupt et al., 1991; Liu et al., 1990), and some moderate AD patients may not acknowledge familiar faces (Förstl and Kurz, 1999). Approximately one third of moderate Alzheimer's patients develop delusional symptoms triggered by the progressing cognitive deficits (Förstl et al., 1993; Reisberg et al., 1996).

Patients in this stage often lack emotional control and develop temper tantrums accompanied by physical or verbal aggression (Förstl and Kurz, 1999). Behavior like wandering and hoarding are characteristic of moderate AD (Devanand et al., 1997). Patients in the moderate stage of AD require constant supervision. Certain household appliances, such as a

gas stove or a toaster, are a constant source of danger (Haupt and Kurz, 1993; Stern et al., 1997). Many patients are at an increased risk of falls due to an inconsistent walking pattern and a stooped posture (Förstl et al., 1992).

1.1.4 Severe

In the late stage of the illness, even early biographical memories can be lost (Förstl and Kurz, 1999). Patients communicate by simple phrases or single words, and it is a challenge to express even the simplest of needs (Förstl and Kurz, 1999). While language skills are severely reduced, many can comprehend and convey emotional signals. This causes patients to often misunderstand interventions, and subsequently generate panic and anger (Förstl and Kurz, 1999). A significant fraction of patients with severe AD displays extreme apathy and exhaustion.

Individuals require support while eating and drinking, where double incontinence is commonplace (Franssen et al., 1993). Comorbidity (the presence of another disease) with Parkinson's disease is common, and often causes an inability to initiate movement and remain motionless (Förstl and Kurz, 1999). Jerky muscle contractions and epileptic seizures may be observed in a small fraction of severe AD patients (Förstl et al., 1992; Romanelli et al., 1990). Pneumonia and the obstruction of the blood supply to the heart (myocardial infarction) are the most common causes of death in AD (Förstl and Kurz, 1999).

1.2 Pathophysiology

1.2.1 Biochemistry

Alzheimer's disease is a protein misfolding disease caused by the accumulation of misfolded beta-amyloid (Aβ) and tau proteins in the brain (Hashimoto et al., 2003). These accumulated Aβ proteins form plaques that are made up of small peptides approximately 39-43 amino acids in length (Hashimoto et al., 2003). Beta-amyloid fibrils are generated when the large amyloid precursor protein (APP) is cleaved by enzymes through proteolysis (Hooper, 2005). These fibrils form deposits outside of the nerve cells, called amyloid plaques (Ohnishi and

Takano, 2004). In addition to beta-amyloid aggregation, tau protein also abnormally accumulates. The cytoskeleton of nerve cells is partially supported by internal microtubules. The microtubules transport essential nutrients and molecules to and from the cell body to the axon. In healthy neurons, tau stabilizes the microtubules (Hernández and Avila, 2007) (see Figure 1.1).

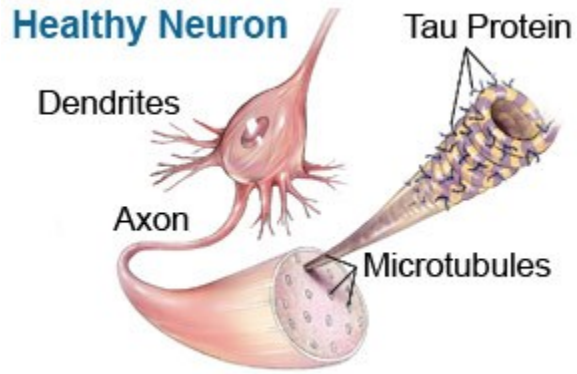

Figure 1.1. Healthy neuron showing the normal tau protein (BrightFocus Foundation, 2012).

When tau protein becomes hyperphosphorylated, it joins with other tau proteins to create neurofibrillary tangles and destroys the neuron's transport system (Hernández and Avila, 2007).

1.2.2 Neuropathology

Alzheimer's disease is described by the loss of neurons and synapses (neurodegeneration) in the cerebral cortex and other subcortical regions of the brain which results in significant atrophy of the affect regions (Wenk, 2003). Beta-amyloid plaques and neurofibrillary tangles are observed by microscopy in AD brains (Tiraboschi et al., 2004). Plaques are dense, mostly insoluble aggregates of beta-amyloid peptides outside and around nerve cells where tangles are masses of hyperphosphorylated tau protein that accumulate inside the neurons. While plaques and tangles are developed as a part of the ageing process, those with Alzheimer's generally have a larger number of plaques and tangles in specific brain regions (Bouras et al., 1994). Additionally, abnormal protein aggregates found in Parkinson's disease, referred to as Lewy bodies, are often found in AD brains (Kotzbauer et al., 2001).

1.2.3 Genetics

Only a small portion of Alzheimer's patients are genetically inherited although there are some genes that may act as risk factors for the majority of Alzheimer's cases (Khachaturian, 1985). Familial Alzheimer's disease (FAD) is inherited by autosomal dominance as opposed to sex-linked inheritance. This means that if the abnormal gene is inherited from either parent, the offspring will display the abnormal gene. Those with FAD usually show signs of AD before the age of 65 (Blennow et al., 2006). The abnormal gene linked to FAD is a mutation of one of three genes: the amyloid precursor protein, or presenilins 1 or 2 (Waring and Rosenberg, 2008). The mutation in APP or the presenilin genes can create an increase in Aβ42, where 42 is the specific amino acid of the Aβ peptide chain (Selkoe, 1999). The genetic mutation can also vary the ratio between Aβ42 and Aβ40 without specifically increasing the levels of Aβ (Selkoe, 1999; Shioi et al., 2007).

The majority of Alzheimer's cases is not autosomal dominant and is referred to as "sporadic AD". The most widely understood genetic risk factor for AD is the inheritance of the ε4 allele of the apolipoprotein E (APOEε4) (Strittmatter et al., 1993; Mahley et al., 2006). Geneticists agree that there are many other genes that may be risk factors for the development of Alzheimer's disease (Waring and Rosenberg, 2008); however, of the nearly 500 genes that have been tested for association with late-onset sporadic AD, all have been deemed insignificant (Blennow et al., 2006; Waring and Rosenberg, 2008).

1.3 Disease Mechanism

The cause of most Alzheimer's cases is still unknown, where a mere 1% to 5% of AD cases have been traced back to genetic differences (Khachaturian, 1985). There are three leading hypotheses that explain Alzheimer's disease: 1) the cholinergic hypothesis, the amyloid hypothesis, and the tau hypothesis (Perry, 1986; Tanzi and Bertram, 2005; Mandelkow and Mandelkow, 1998).

1.3.1 Cholinergic Effect

The cholinergic hypothesis is the original theory behind the cause of AD where most of the available drug therapies are based on this hypothesis (Francis et al., 1999). This hypothesis proposes that the disease is a result of reduced synthesis of the neurotransmitter acetylcholine in nerve cells, hence cholinergic (Francis et al., 1999) (see Figure 1.2). Acetylcholine can either promote a signal between neurons or inhibit a signal, depending upon the type of receptor on the adjoining cell (McDowall, 2013).

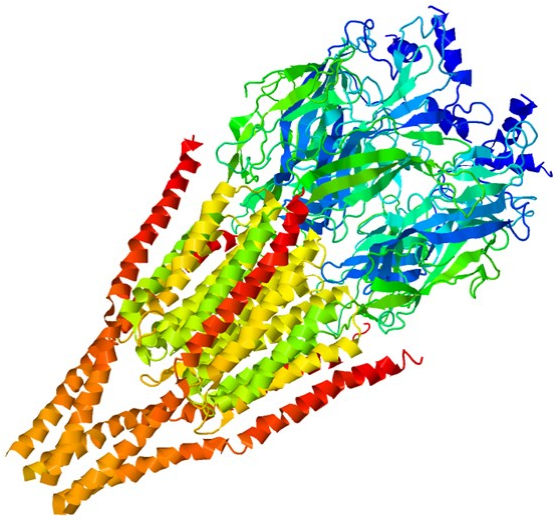

Figure 1.2. 3D structure of the acetylcholine receptor (McDowall, 2013).

The cholinergic hypothesis has not achieved widespread support due to the fact that the medications intended to treat acetylcholine deficiency have not proven effective (Francis et al., 1999). While the initial cholinergic hypothesis has not been accepted, it is the basis of another leading hypothesis: the large-scale aggregation of amyloid protein (Shen, 2004) which promotes generalized neuroinflammation (Wenk, 2003).

1.3.2 Amyloid Protein

The amyloid hypothesis proposes that the sticky beta-amyloid protein misfolds and aggregates to form deposits, or plaques, which cause Alzheimer's disease (Hardy and Allsop, 1991; Sierks et al., 2011). The amyloid precursor protein gene is located on chromosome 21.

Those with Down Syndrome have an extra gene copy on chromosome 21 where almost all individuals with Down Syndrome are afflicted with AD by 40 years of age (Nistor et al., 2007; Lott and Head, 2005). APOEε4, a specific isoform of the apolipoprotein, is a major genetic risk factor for Alzheimer's. Apolipoproteins aid in the deterioration of beta-amyloid, where APOEε4 does not break down Aβ properly, causing an accumulation of amyloid in the brain (Polvikoski et al., 1995). Further studies have shown that transgenic mice that express a mutant form of the APP gene develop fibrillar amyloid plaques and brain pathology similar to that of Alzheimer's disease with spatial learning deficits (Games et al., 1995; Masliah et al., 1996; Hsiao et al., 1996; Lalonde et al., 2002). While an experimental vaccine was found to clear the amyloid plaques from the brain in early human trials, it did not resolve the dementia (Holmes et al., 2008).

Research studies have suggested that beta-amyloid oligomers (e.g., monomers, dimers) are the primary pathogenic form of Aβ (Sierks et al., 2011; Kasturirangan et al., 2012). Amyloid-derived ligands (ADDLs), or the key toxic oligomers, bind to a neuron surface receptor. The bound oligomer subsequently alters the synapse structure and disrupts neuronal communication (Lacor et al., 2007). Other research postulates that another protein similar to Aβ may be the cause of the disease. An amyloid-related process that functions to remove unnecessary neuronal connections in the brain during development may be activated by processes later in life to cause the characteristic neurodegeneration of Alzheimer's disease (Nikolaev et al., 2009). A fragment of APP from the peptide's N-terminus, referred to as N-APP, is adjacent to beta-amyloid and is cleaved from APP. The cleaved N-APP triggers the self-destruct pathway by binding to the neuronal death receptor 6 (DR6) (Nikolaev et al., 2009). DR6 is broadly expressed by developing neurons, and is required for normal cell body death; however, DR6 is also highly expressed in the regions of the brain most affected by AD (Nikolaev et al., 2009). It is feasible that the N-APP/DR6 mechanism is hijacked in Alzheimer's patients to cause neuron damage, where beta-amyloid assists the N-APP/DR6 pathway by suppressing synaptic function (Nikolaev et al., 2009). This thesis focuses on the amyloid hypothesis, specifically the toxic Aβ oligomers that misfold and accumulate to create the beta-amyloid plaques, which are the end state of the pathology.

1.3.3 Tau Protein

The tau hypothesis postulates that tau protein aberrations (see Figure 1.3) trigger the disease pathology (Mudher and Lovestone, 2002).

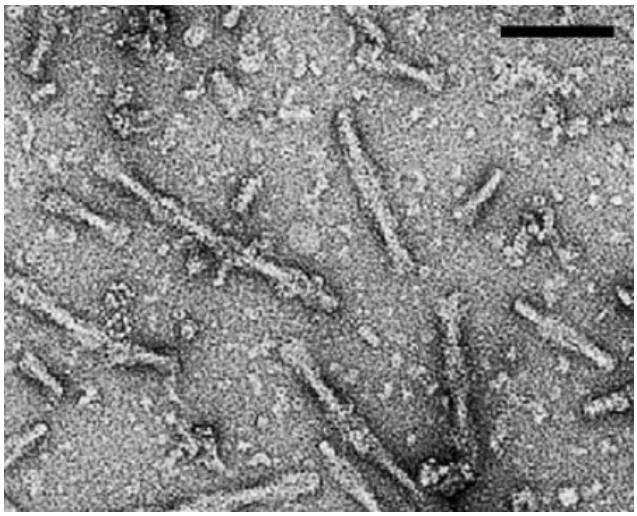

Figure 1.3. Electron micrograph of a negatively-stained preparation of tau filaments of the Alzheimer-type. Scale bar, 100 nm (Goedert et al., 1991).

Hyperphosphorylated tau binds with other tau protein fragments to form neurofibrillary tangles inside nerve cell bodies (Goedert et al., 1991). The neurofibrillary tangles inside the neuron bodies eventually collapse the neuron's transport system by disintegrating the microtubules, or the structural network within the cell's cytoplasm (Igbal et al., 2005). This degeneration of the microtubules is likely the cause of the defective neuronal communication and the cell death (Chun and Johnson, 2007).

1.4 Diagnosis and Treatment

Alzheimer's disease is diagnosed using the patient history and clinical observations (Mendez, 2006; Klafki et al., 2006). Additionally, AD is clinically diagnosed by the presence of amyloid plaques and neurofibrillary tangles in the brain tissue; however the pathogenesis of this devastating disease occurs decades before the first symptoms manifest (Price and Morris, 1999; Braak and Braak, 1997). Medical imaging like magnetic resonance imaging (MRI) or positron

emission tomography (PET) scans can be employed to verify the presence of Alzheimer's disease (Caselli and Reiman, 2013). Medical imaging may also predict the progression from mild cognitive impairment to moderate Alzheimer's disease (Schroeter et al., 2009) thereby indicating the disease stage (Waldemar et al., 2007). Diagnosis of AD can be confirmed by a post-mortem brain biopsy (McKhann et al., 1984).

Notable advances in the understanding of Alzheimer's disease have been established over the past 25 years (Selkoe, 2011; Karran et al., 2011; De Leon et al., 2004; Kasturirangan et al., 2012; Saunders et al., 1993; Pfeffer et al., 1987); however, applicable treatments and prevention measures have yet to be established. The 2010 annual economic cost of those diagnosed with Alzheimer's disease in the United States was estimated at more than $172 billion (Alzheimer's Association, 2010), and is expected to rise to a trillion dollars by 2050 without the development of disease-altering treatments (Brookmeyer et al., 2011).

1.5 Biomarkers as a Diagnostic Tool

An efficient set of biomarkers would allow for the study of toxic mechanisms and metabolic pathways that cause Alzheimer's disease and would eventually lead to an early diagnostic method (Sierks et al., 2011). Comprehensive longitudinal studies of AD biomarkers require an extended period of time to describe the gradual pathologic degradation that ultimately leads to dementia. Two popular, potential biomarker methods for Alzheimer's are those found in cerebrospinal fluid and blood plasma (Quinn, 2013).

1.5.1 Cerebrospinal Fluid Biomarkers

Amyloid-beta 42 (Aβ42), total tau, (t-tau or tau), and phosphorylated tau (p-tau) comprise the focus of cerebrospinal fluid (CSF) biomarker research as these proteins are the metabolites of neuropathological changes (Allan et al., 2010). In studies that compare AD patients with nondemented controls, there is a high sensitivity of 81% and specificity of 91% for t-tau and p-tau and 86% sensitivity and 89% specificity for Aβ42 (Blennow, 2004). From these studies, it has been determined that the presence of low CSF Aβ42 and high tau levels accurately predicts the

manifestation of pathological characteristics of post-mortem Alzheimer's disease (Tapiola et al., 2009).

1.5.1.1 Amyloid Beta Peptides

The small amyloid beta (Aβ) peptide fragments (Aβ42 and Aβ40) are produced by the proteolytic cleavage of a large transmembrane protein that resides in the brain, amyloid precursor protein (APP) (Marchesi, 2012). While the functions of APP and the cleaved peptides are unknown, it is certain that each influences the operations of the brain (Marchesi, 2012). The AD plaques are primarily composed of Aβ peptides, where the majority of these peptides contain 42 amino acids (Aβ42) (Ikeda et al., 1989). Aβ42 is traditionally secreted by healthy cells (Haass et al., 1992), but genetic mutations instigate the overproduction of Aβ42 which results in familial AD (Hardy and Selkoe, 2002). A collection of experimental data and clinical observations demonstrates that Aβ peptides are the primary cause of Alzheimer's dementia; this popular theory is known as the Amyloid Hypothesis (Marchesi, 2012).

Multiple studies have illustrated a decrease of Aβ42 in the CSF by approximately 50% in individuals with AD compared to cognitively normal controls of the same age with the diagnostic sensitivity and specificity ranging from 80 to 90% (Blennow and Hampel, 2003; Formichi et al., 2006). One longitudinal AD study demonstrated that Aβ42 continually decreases with the progression of the disease (Tapiola et al., 2000). When CSF Aβ42 was analyzed exclusively, the specificity level was roughly 55%; however, when considered with tau protein, the specificity of the combined test was 86% (Hampel et al., 2008). A 2003 autopsy study revealed the strong inverse correlation between CSF Aβ42 levels and the quantity of fibrillary amyloid deposition in plaques (Strozyk et al., 2003). It is hypothesized that with increasing age, amyloid plaques accumulate in the brain and act as a sink for soluble Aβ (DeMattos et al., 2001).

1.5.1.2 Total Tau Protein

Tau is a natively unfolded protein that stabilizes microtubules and is naturally found in neurons. Tau aggregates comprise the neurofibrillary tangles characteristic of AD (Mandelkow

and Mandelkow, 2012), and consequently, the tau protein is of major interest as a CSF biomarker. While t-tau is highly soluble, pathological aggregation occurs via short, hydrophobic motifs forming β structures (Mandelkow and Mandelkow, 2012). The aggregation of tau protein is toxic in cell and animal models, but can be broken down by aggregation inhibitors or by suppressing expression (Mandelkow and Mandelkow, 2012).

Longitudinal experiments have shown that patients with AD have approximately a 300% increase in tau concentration compared to that of a nondemented subject, and can anticipate an incremental increase in t-tau levels over a lifespan (Popp et al., 2010). The sensitivity and specificity of such studies range between 80 and 90% (Blennow and Hampel, 2003). The results from various studies suggest that CSF tau concentrations indicate the degree of neuronal and axonal degeneration and damage in the brain (Ost et al., 2006; Hesse et al., 2001). Additionally, high CSF tau levels have been link to the rapid progression from MCI to Alzheimer's dementia (Blom et al., 2009). However, clinical studies have shown that elevated CSF tau concentrations are not specific to AD as they are similarly increased in other neurodegenerative diseases (Arai et al., 1997).

1.5.1.3 Hyperphosphorylated Tau Protein

P-tau is the result of the abnormal hyperphosphorylation of total tau at many sites (Craig-Schapiro et al., 2009). Due to the irregular phosphorylation, tau cannot bind and stabilize microtubules, potentially leading to axon deterioration (Mandelkow and Mandelkow, 1998). Approximately thirty phosphorylation epitopes have been discovered in AD patients where the majority of the studies have examined p-tau at threonine 231 (p-tau231P) and threonine 181 (p-tau181P) (Hampel et al., 2008). Many studies have shown that CSF p-tau levels are increased in AD patients (Andreasen et al., 1998; Burger nee Buch, et al., 1999; Csernansky et al., 2002; Galasko et al., 1998; Skoog et al., 1995), where roughly 20 experiments consisting of approximately 2,000 patients and controls boast a sensitivity and specificity between 80 and 90% (Formichi et al., 2006; Hampel et al., 2008). One explanation for this elevated p-tau concentration is that degenerating neurons release p-tau which subsequently diffuses into the

CSF (Mandelkow and Mandelkow, 1998). Levels of p-tau in the cerebrospinal fluid seem to suggest the phosphorylation state of tau and the formation of neurofibrillary tangles in the human brain (Biagioni and Galvin, 2011).

1.5.1.4 Oligomeric Amyloid Beta and Tau Protein

While CSF Aβ42 and tau show promise as diagnostic biomarkers for Alzheimer's disease, more selective diagnostic biomarkers may be obtained by detecting specific toxic protein species associated with AD (Sierks et al., 2011). The precise detection and measurement of the levels of each relevant toxic protein species (those of Aβ, t-tau, p-tau) may offer a method to study the mechanisms of toxicity and progression of the disease. Furthermore, the identification and quantification of these toxic forms may lead to the presympotmatic diagnosis of Alzheimer's disease (Sierks et al., 2011). It has been shown that various diverse soluble Aβ and tau species correlate well with the progression of AD (Lue et al., 1999; McLean et al., 1999; Maeda et al., 2006; Bruden et al., 2008; Meraz-Rios et al., 2010), whereas the presence of amyloid plaques does not (Knopman et al., 2003; Braak and Braak, 1998).

Specific soluble aggregate morphologies of Aβ, tau, and p-tau are likely present at very low concentrations in CSF, thus highly selective reagents that explicitly identify each of the target species are required (Sierks et al., 2011). The imaging abilities of atomic force microscopy and the binding diversity of bacteriophage display antibody technology have been utilized in conjunction as a novel biopanning technique to isolate reagents (A4, E1 nanobodies) (Kasturirangan et al., 2012) that bind to specific oligomers of amyloid beta (Sierks et al., 2011). The identified reagents can be employed to determine if specific aggregate forms of Aβ and tau can be detected in CSF and if these morphologies hold promise to diagnose AD (Sierks et al., 2011). This thesis focuses on the use of selective reagents created in the laboratory to target specific morphologies of amyloid beta.

1.5.2 Plasma Biomarkers

Effective plasma markers, detected through venipuncture, are of major interest due to their potential as a truly minimally invasive test (Biagioni and Galvin, 2011). Unfortunately, research to use plasma Aβ as a biomarker for Alzheimer's dementia has proven ineffective (Roher et al., 2009). Innovative molecular techniques have isolated plasma proteins (IL-1alpha, IL-3, TNF-alpha) which can differentiate AD from nondemented controls with almost 90% accuracy, and can distinguish patients with MCI who progress to AD (Ray et al., 2007). While the rapid development of technology promotes the diagnostic accuracy and applicability of plasma biomarkers, these noninvasive markers currently reside in research.

1.6 Thesis Objectives

There exists an imperative need to develop a non-invasive test for the earlier detection of Alzheimer's disease. The use of biomarkers is a promising option that examines the toxic mechanisms and metabolic pathways that cause Alzheimer's disease, eventually leading to an early diagnostic method. This thesis presents the use of oligomeric beta-amyloid as a biomarker to detect Alzheimer's disease via an enzyme-linked protein assay. Amyloid beta protein is isolated through the use of the A4 capture antibody, which has been characterized and identified for its binding specificity to Aβ oligomers (Kasturirangan et al., 2012). The objective of this thesis is to optimize a specialized A4 capture protein assay and quantify the amount of beta-amyloid oligomers in various mice samples using the perfected protein assay.

This thesis begins with the background of Alzheimer's disease and the importance of an earlier diagnostic tool. The protein assay protocol development and materials are expanded upon in Chapter 2, and the results and discussion of this work appear in Chapter 3. Chapter 4 concludes this thesis with final remarks and recommendations for future work.

CHAPTER 2

DEVELOPMENT OF PHAGE CAPTURE ELISA TO DIFFERENTIATE BETWEEN NEURODEGENERATIVE DISEASES

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2.1 Introduction

Any disorder caused by the gradual and progressive loss of neural tissue in the central nervous system can be classified as a neurodegenerative disease. Neurodegenerative diseases are specifically associated with the atrophy of the affected central or peripheral nervous system structures (Bahk et al., 2013). The presence of protein aggregates, called inclusion bodies or Lewy bodies, caused by the misfolding and subsequent aggregation of abnormal proteins is a pathogenic hallmark of neurodegenerative diseases. Alzheimer's disease (AD), the most prevalent neurodegenerative disease which affects more than 5 million United States residents (Bateman et al., 2012), is caused by the accumulation of misfolded beta-amyloid (Aβ) and tau proteins in the brain (Hashimoto et al., 2003). Similarly, aggregated alpha-synuclein (a-syn) is a major component of the Lewy bodies associated with Parkinson's disease (PD) (Dauer and Przedborski, 2003; Spillantini et al., 1997). AD, PD, Huntington's disease, amyotrophic lateral sclerosis (ALS), and prion diseases each possess common cellular and molecular mechanisms including abnormal protein aggregation and the formation of inclusion bodies (Ross and Poirier, 2004). An emerging theory states that the Lewy bodies and protein masses collectively signify an end state of the pathology, where the pathogenesis occurs prior to the formation of these aggregates (Ross and Poirier, 2004; Sierks et al., 2011; Kasturirangan et al., 2012).

Notable advances in the understanding of neurodegenerative diseases have been established over the past 25 years (Prusiner, 1987; Simonian and Coyle, 1996; McGeer and McGeer, 1995; Dexter et al., 1991; Gusella and MacDonald, 2000; Feng and Gao, 2012); however, applicable treatments and prevention measures have yet to be established. Currently, 5 million Americans suffer from Alzheimer's disease, 1 million from Parkinson's, 30,000 from ALS, and 30,000 from Huntington's disease (Harvard NeuroDiscovery Center, 2013). Without the development of disease-modifying therapies, more than 12 million Americans will suffer from neurodegenerative diseases by 2040 (Harvard NeuroDiscovery Center, 2013). There exists a need to develop a method of earlier disease detection, specifically the presymptomatic detection of neuronal dysfunction for neurodegenerative diseases (Henley et al., 2005). In this effort, sensitive detection methods are required to assess disease progression.

A biomarker is a traceable substance that indicates the presence or degree of a biological or pathogenic process (Henley et al., 2005). Biomarkers are particularly applicable to neurodegenerative diseases as they can provide an early diagnosis at a stage when disease-altering treatments are expected to be most effective. Treating the disease during this initial stage has the potential to circumvent the devastating neurodegeneration characteristic of these diseases. Analogously, biomarkers can monitor the efficiency of a therapy through disease progression (Henley et al., 2005). A sensitive method of detection for disease-specific biomarkers that are present during pathogenesis would allow for the study of the toxic mechanisms and metabolic pathways that cause the neurodegenerative diseases and would eventually lead to an early diagnostic method (Sierks et al., 2011). This paper details the optimization and development of a novel phage capture ELISA (enzyme-linked immunosorbent assay) that can detect the relatively quantity of a disease-specific antigen.

2.2 Materials and Methods

2.2.1 Production and Purification of scFvs

Each neurodegenerative disease has a specific target antigen to which a specialized scFv will bind. These scFvs which bind to various oligomeric morphologies of the target antigen were identified and produced in lab. A colony with the scFv was grown overnight in 1.55 grams of 2xYT, 50 mL of distilled water, 2.5 mL of 20% glucose, and 50 µL of ampicillin at 37°C. The overnight culture was utilized to create an expression culture containing 31 g of 2xYT, 1 liter of distilled water, 10 mL of 20% glucose, 1 mL of ampicillin, and 10 mL of the overnight culture. The expression culture incubated on a shaker at 30°C until the absorbance optical density at 600 nm (OD_{600}) was approximately 0.8. Once this OD_{600} was achieved, expression was induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG). The scFv remained in the shaker at 37°C for at least 12 hours.

The scFvs were purified using Fast Protein Liquid Chromatography (FPLC). The supernatant from a one liter bacterial culture was filtered using a 0.2 µm filter (Whatman, Clifton,

NJ), and concentrated with a tangential flow filter (Milipore) using a 10 kDa filter membrane (Millipore, Billerica, MA). The concentrated scFvs were applied to a protein A-Sepharose column (GE healthcare, NJ) which was equilibrated in 1X PBS, pH 7.4, at 4°C. After washing the column with the 1X PBS buffer, the bound scFv was eluted from the column with 0.2 M glycin, pH 3. The liquid fractions containing the scFv were pooled, adjusted to neutral pH, dialyzed, and stored at -20°C. The purity of the scFv was estimated by electrophoresis on 12% (W/V) SDS-polyacrylamide gels and by a Western blot. The quantity of the scFvs was estimated by Bicinchoninic Acid assay (BCA assay).

2.2.2 Brain Tissue Homogenization

Brain homogenization buffer was prepared with 50 mM Tris and 5 mM EDTA at pH 7.5. 1 mL of brain homogenization buffer was added to the tissue and the tissue was manually chopped with a razor blade to break down any agglomerates. This tissue mixture was pipetted into a 1.5 mL centrifuge tube along with an additional 0.5 mL of brain homogenization buffer. The mixture was sonicated on ice with 10 second bursts followed by 15 second pauses for a total of 5 minutes using a Fisher sonic dismembrator. All of the samples were centrifuged at 13,000 rpm in a Sorvall SLA-1500 rotor at 4°C for 20 minutes. The supernatants were stored at -80°C.

2.2.3 Conditions of Primary Capture scFv

Varying concentrations of each scFv diluted in PBS were added to a high-binding ELISA plate. The incubation temperatures and durations were also varied. The plates were washed three times with 0.1% PBS-Tween after the scFv was discarded. The total protein remaining in each well was measured using a BCA kit (Pierce, USA).

2.2.4 Phage Production

Bacteriophage, often called phage, was produced using the protocol described in the Human Single Fold scFv Libraries I and J manual (MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering (Cambridge, UK)). The selected clones were grown

overnight in 2xYT containing 100 µg/mL ampicillin and 1% glucose. A 1:100 dilution of each clone was grown until the OD_{600} was between 0.4 and 0.6. 2×10^{11} of KM13 helper phage was added to the culture before incubating at 37°C for 30 minutes without shaking. After spinning at 3,000 g for 10 minutes, the pellet was resuspended in 2xYT containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 0.1% glucose. The suspended pellet was cultured at 30°C overnight. The culture was centrifuged for 30 minutes at 3,000 g and PEG/NaCl was added to the supernatant. This solution incubated on ice for 1 hour. The solution was spun, the supernatant discarded, and the pellet re-spun briefly. The pellet was then resuspended in PBS, spun at 11,600 g, and the resultant supernatant stored in glycerol. The phage was titered using TG1 cells to determine its concentration. The phages bound to all forms of their target proteins, including monomeric and oligomeric morphologies, making these phages ideal proteins for the ELISA detection antibodies.

2.2.5 Biotinylation Protocol

Three different types of biotinylation were employed to biotinylate the bacteriophage where samples with varying millimoles of biotin were tested to achieve the largest ELISA signal. The first type of biotin utilized was the Sulfo-NHS biotinylation (amine biotinylation) using the EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Scientific, USA). The Sulfo-NHS biotin was added to the phage and allotted 2 hours to incubate on ice. The excess biotin was then removed using a Zeba spin desalting column (Thermo Scientific, USA). The second type of biotinylation (carboxyl biotinylation) was performed using the EZ-Link Pentylamine-Biotinylation kit (Thermo Scientific, USA). The third biotinylation method was performed using the EZ-Link Pentylamine-Biotinylation kit with the addition of Sulfo-NHS to the reaction. It was proposed that the addition of Sulfo-NHS to the Pentylamine Biotin may increase the yield of biotinylated phage. One of the antigen-specific scFvs was also biotinylated using the amine biotin.

In order to determine if the bacteriophage was indeed biotinylated, a modified ELISA procedure was used. The biotinylated phage was added to an ELISA plate, followed by blocking solution (Bovine Serum Albumin (BSA) or non-fat dry milk) and avidin/HRP (Sigma-Aldrich, USA).

The plate was visualized using 3,3′,5,5′-Tetramethylbenzidine (TMB) at 605 nm on a Wallac Victor^2 microplate reader.

2.2.6 Determination of the Optimum Biotinylated Phage, Blocking Conditions, and Phage Concentration

The purified target antibodies, such as alpha-synuclein or beta-amyloid, that may play a role in the various neurodegenerative diseases, were given sufficient time to aggregate at 37°C. These proteins were used as target antigens in indirect ELISAs to determine which of the biotinylated phages produced the largest ELISA signal and which blocking solution was the most effective.

The ELISA plates were coated with aggregated target proteins, and then blocked at 37°C. The biotinylated phage was added to the plate where the phage binds to all morphologies of the target protein including monomers and oligomers. Avidin/HRP was then added to the plate. The plate was washed three times between each step and the ELISA was visualized using TMB. The best blocking condition was determined using the target antigen followed by numerous blocking solutions. The superior blocking solution was selected and used with varying concentrations of the different biotinylated phages to confirm the ideal phage dilution. Lastly, an indirect ELISA was performed to compare the phages with their target antigen and optimized blocking solution to identify the most promising biotinylated bacteriophage.

2.2.7 Titration of Target Proteins Using Indirect ELISA and TMB

An indirect ELISA was executed in a similar fashion as described in the previous section. The aggregated target antigen was diluted from nanomolar to picomolar concentrations to determine the lowest concentration that can readily be identified by the biotinylated phage. TMB was used as the visualization reagent.

2.2.8 Phage Capture ELISA using TMB

The capture scFv was added to the wells of a high-binding ELISA plate. The plate was washed three times with 0.1% PBS-Tween. Blocking solution was added to the wells and the wash protocol was repeated. The brain samples were added to the wells. The biotinylated phage was added to wells followed by avidin/HRP. After the phage and avidin/HRP steps, the plate was washed four times with 0.1% PBS-Tween. The plate was visualized using TMB. Results were presented as signal ratio of sample with antigen target to the control without antigen target, that is, the absorbance from the wells with samples was divided by that from the wells without samples.

2.2.9 Phage Capture ELISA using Chemiluminescent Kit

The ELISA protocol was repeated as detailed in Section 2.2.8 with the exception of the visualization reagent. In this ELISA protocol, the plates were visualized using either the SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific, USA) or the SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) kit. Results were presented as a ratio of the signal of the sample to the signal of the control without sample.

2.2.10 Normalized Relative Concentrations to Measure Oligomer Levels

In order to create a standard to measure the relative concentration of oligomeric morphologies in the samples tested, the sample with the greatest binding affinity to the scFv of interest was used. This sample was diluted to 1:50, 1:100, 1:200, 1:500, 1:1,000, and 1:2,000 in PBS and a standard curve was generated. A linear regression was determined for the standard curve and this linear model was used to determine the relative dilution of the samples. Each sample was normalized by dividing its relative dilution by the average relative dilution of the control without sample.

CHAPTER 3

DETECTING BETA-AMYLOID OLIGOMERS IN TRIPLE-TRANSGENIC AND NONTRANSGENIC MICE

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Abstract

The popular amyloid hypothesis states that beta-amyloid protein aggregates to form sticky deposits in the brain tissue, causing Alzheimer's disease. Current research studies propose that oligomeric morphologies of beta-amyloid are the principal pathogenic form of the protein. Through the use of a highly-specified capture enzyme-linked immunosorbent assay, the relative quantity of beta-amyloid oligomers was determined in triplicate for a total of 16 transgenic (TG) and nontransgenic (NTG) mice aged five, nine, and thirteen months. A significant p-value of 0.000212 was found between the TG and NTG five month mice. As the age of the TG mice increased, the relative quantity of beta-amyloid oligomers decreased. This suggests that the beta-amyloid oligomers were aggregating to form beta-amyloid fibrils. Conversely, as the age of the NTG mice increased, the quantity of beta-amyloid oligomers increased. This finding supports previous studies that have shown the production of beta-amyloid oligomers in normal aging.

3.1 Introduction

Alzheimer's disease (AD) is the leading neurodegenerative disease, affecting roughly 8% of people 65 years of age or older (Small et al., 1997). Alzheimer's disease is clinically diagnosed by widespread neurodegeneration with the presence of neurofibrillary tangles and beta-amyloid plaques in the brain (McKhann et al., 1984); however, a definitive diagnosis of this disease requires histopathological evidence by autopsy or brain biopsy (Shim and Morris, 2011). AD is preceded by gradual, progressive memory loss that is typically characterized by Mild-Cognitive Impairment (MCI) (Petersen et al., 2001; Winblad et al., 2004). The presence of amyloid plaques and neurofibrillary tangles, the neuropathological hallmarks of Alzheimer's disease, has been reported in adults without dementia, indicating that the pathogenesis that causes AD occurs years before any symptoms develop (Bookheimer et al., 2000). Current treatments for dementia aim to slow the progression of the disease rather than regenerate neurons, establishing the necessity to identify early stage markers of cognitive decline (Bookheimer et al., 2000).

Notable advances in the understanding of Alzheimer's disease have been established over the past 25 years (Selkoe, 2011; Karran et al., 2011; De Leon et al., 2004; Kasturirangan et al., 2012; Saunders et al., 1993; Pfeffer et al., 1987); however, applicable treatments and prevention measures have yet to be determined. Without the development of disease-altering treatments, the number of people 65 years of age or older with Alzheimer's disease is projected to reach 7.1 million by the year 2025, a 40% increase from the 5 million people age 65 years and older currently affected (Alzheimer's Association, 2013). A method of earlier disease detection is essential, specifically presymptomatic detection during the initial, pathogenic stages where early detection has the potential to prevent the neuronal deterioration (Henley et al., 2005).

A biomarker is a traceable substance that indicates the presence or degree of a biological or pathogenic process (Henley et al., 2005). An efficient biomarker is particularly applicable to Alzheimer's disease as it can provide an early diagnosis at a stage when disease-altering treatments are expected to be most effective. Additionally, an AD biomarker can monitor the efficiency of a therapy through disease progression (Henley et al., 2005). A sensitive method

of detection for an Alzheimer's disease biomarker that is present in the presymptomatic stages would allow for the study of the toxic mechanisms and metabolic pathways that cause the neurodegeneration and would eventually lead to an early diagnostic method (Sierks et al., 2011).

The amyloid hypothesis proposes that the sticky beta-amyloid protein misfolds and aggregates to form deposits, or plaques, which cause Alzheimer's disease (Hardy and Allsop, 1991; Sierks et al., 2011). Current research studies have indicated that beta-amyloid oligomers (e.g., monomers, dimers) are the primary pathogenic form of Aβ (Sierks et al., 2011; Kasturirangan et al., 2012). This chapter illustrates the application of a phage capture ELISA (enzyme-linked immunosorbent assay) that was optimized to detect picomolar concentrations of beta-amyloid oligomers in a given triple-transgenic mice brain tissue sample. A mouse model was chosen to analyze the disease progression because triple-transgenic mice overexpress beta-amyloid and thus provide an ideal template to test the optimized ELISA.

3.2 Methods

3.2.1 Phage Capture ELISA

An optimized enzyme-linked immunosorbent assay (ELISA) was employed to detect the presence of beta-amyloid oligomers, a promising biomarker for Alzheimer's disease, in each mice brain tissue sample. The capture ELISA utilized an eight by twelve, Corning® 96 well EIA/RIA high-binding plate to create a chain of hyper-specific binding antibodies (see Figure 3.1).

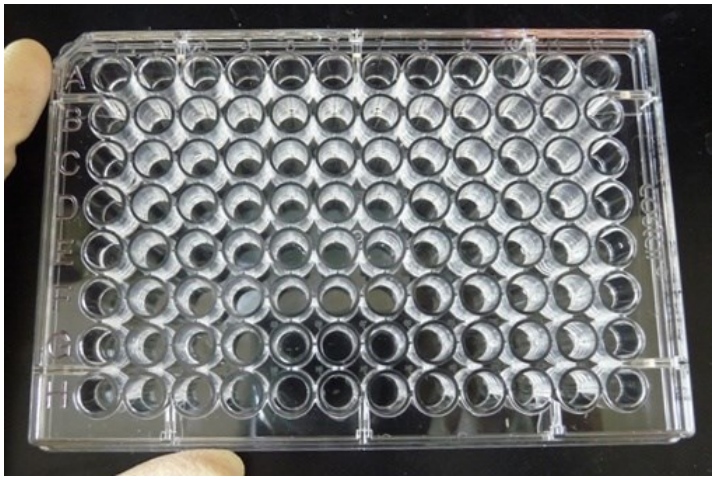

Figure 3.1. A 96 well high-binding ELISA plate.

The end of the antibody chain reacts with a substrate to cause a color change. The optical density of the color can be used to identify the relative quantity of a specific antigen by a plate reader. The ELISAs summarized in this chapter were optimized to exclusively detect the oligomeric beta-amyloid protein biomarker in each sample.

The first step in the ELISA protocol is to add the capture antibody, called a single-chain variable fragment (scFv), to the high-binding plate, at a concentration of 0.3 mg/mL. This concentration was determined sufficient to saturate the plate and ensure a significant detection signal. The scFv utilized in this research project was an antibody created in-house called A4 (refer to Section 2.2.1 in Chapter 2), which incubated on the plate at 37°C for two hours. A4 was chosen due to its binding specificity for beta-amyloid oligomers (Kasturirangan et al., 2012). The plate was then washed three times with 0.1% OmniPur polyoxyethylene (20) monolaurate (Tween 20) in a 1X, 7.4 pH phosphate buffered saline (PBS) wash solution to remove any unbound A4. The plate was blocked with 2% non-fat dry milk in 1X PBS for an hour at 37°C to prevent any non-specific proteins from binding to the plate. After removing the milk and washing the plate, the mice tissue sample was diluted in 1X PBS to 0.2 mg/mL, added to the plate, and allotted two hours at 37°C to bind to the capture A4 scFv.

Again the plate was washed in 0.1% Tween 20 and biotinylated H1V2 bacteriophage, often referred to as phage, was added to the plate as the specific primary antibody for the beta-

amyloid capture ELISA. The H1V2 phage bound to all morphologies of beta-amyloid, including monomers and oligomers, making H1V2 bacteriophage the ideal protein for the ELISA. The phage, created in-house (refer to section 3.3.2 for methods) at a concentration of approximately 0.02 µg/mL, incubated on the plate for an hour at 37°C. The plate was washed four times and 0.5 µg/mL of horseradish peroxidase (HRP)-linked avidin protein was added to the plate as the secondary detection antibody for Aβ. The avidin/HRP incubated at 37°C for an hour and was washed four times. The final step was to utilize the Thermo Scientific Supersignal® ELISA Femto Maximum Sensitivity Substrate (Product # 37074) to visualize the relative quantity of beta-amyloid in each mice brain sample. Using this method, the luminescence of each sample was evaluated twice, after 1 minute and again after 30 minutes, using the Perkin Elmer Wallac Victor^2 1420 Multilabel Counter plate reader and its Wallac 1420 Workstation software (see Figure 3.2).

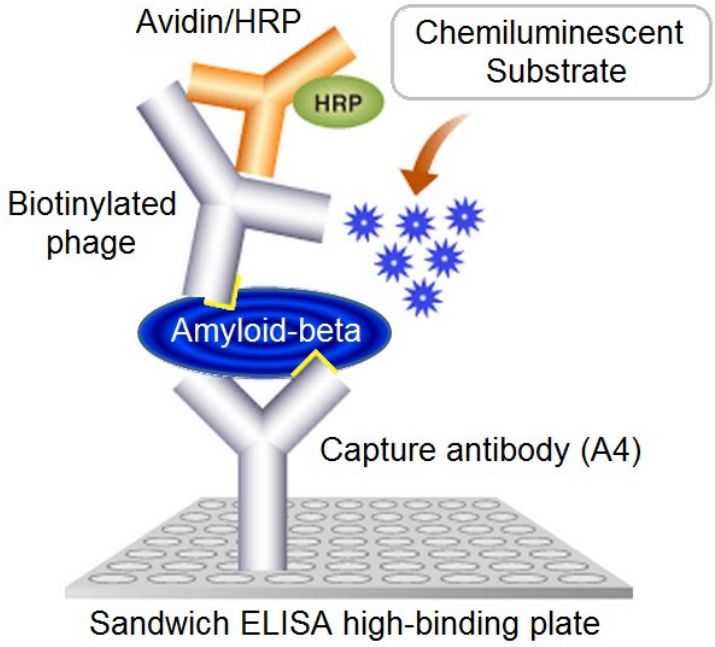

Figure 3.2. Phage capture ELISA (Cell Signaling Technology, 2013).

3.2.2 Bacteriophage Production

The process to generate the H1V2 bacteriophage followed the Tomlinson I and J phage library protocol from Medical Research Council (Cambridge, UK). The H1V2 bacteriophage was

generated using in-house KM13 helper phage and 2xYT expression media. 500 µL of the phage plasmid in E. coli was added to 200 mL of 2xYT containing 100 µg/mL ampicillin and 1% glucose. This was grown in the shaker for more than two hours at 37°C until the OD_{600} was roughly 0.5. KM13 helper phage was added in a 20:1 phage to E. coli ratio and incubated without shaking at 37°C for 30 minutes. The solution was then centrifuged at 3,000 g for 10 minutes. The pellet was resuspended in 100 mL of 2xYT containing 100 µg/mL ampicillin, 50 µg/mL kanamycin, and 0.1% glucose. This incubated at 30°C overnight in a shaker.

After incubating, the H1V2 phage was centrifuged at 3,300 g for 30 minutes, and 25 mL of 20% Polyethylene glycol 6000 and 2.5 M NaCl (PEG/NaCl) was added to and mixed with the supernatant before incubating on ice for one hour. The phage was spun again at 8,000 g for 30 minutes and the PEG/NaCl was removed. The pellet was resuspended in 4 mL of 1X PBS and centrifuged at 11,600 g for 10 minutes. 15% glycerol was added to the phage supernatant and the phage was titered to determine the concentration. Additionally, the phage concentration was determined by a BCA assay (Pierce, USA). The H1V2 bacteriophage was biotinylated using the Thermo Scientific EZ-Link® Pentylamine Biotin Kit, Product Number 21345 (refer to Section 2.2.5 in Chapter 2), so that they would bind to the avidin/HRP on the ELISA.

3.2.3 Statistical Analysis

IBM® SPSS® Statistics Version 21 was employed to analyze the data and determine significance between the means of the samples. An independent samples t-test was chosen to analyze the significance between three sets of two distinct samples. A one-way analysis of variance (one-way ANOVA) was used to compare the means of all six of the samples tested.

3.3 Results

Six different groups of homogenized mice brain tissues were tested via ELISA to measure the relative quantity of beta-amyloid oligomers. The six groups consisted of three triple-transgenic and three nontransgenic (wild type) five month, nine month, and thirteen month old mice samples. The triple-transgenic mice samples were provided by the Translational Genomics

Research Institute (TGen) in Phoenix, Arizona, where these triple-transgenic mice overexpress human amyloid precursor protein, human presenilin protein (PS1), and human tau protein.

The triple-transgenic and nontransgenic five month mice groups each consisted of two mice. The four mice were tested in triplicate via ELISA and the resultant signals were normalized such that the average of the nontransgenic mice group was equal to one. The transgenic group achieved an average signal of 3.62 with a standard error of ± 0.43, accounting for ± 12.0% of the average. The nontransgenic mice had an average signal of 1.09 with a ± 0.11 standard error or ± 10.2% of 1.09. The transgenic group had an average roughly 3.32 times that of the nontransgenic group. It is important to note the asterisk on the TG group which denotes a p-value less than 0.05 (see Figure 3.3).

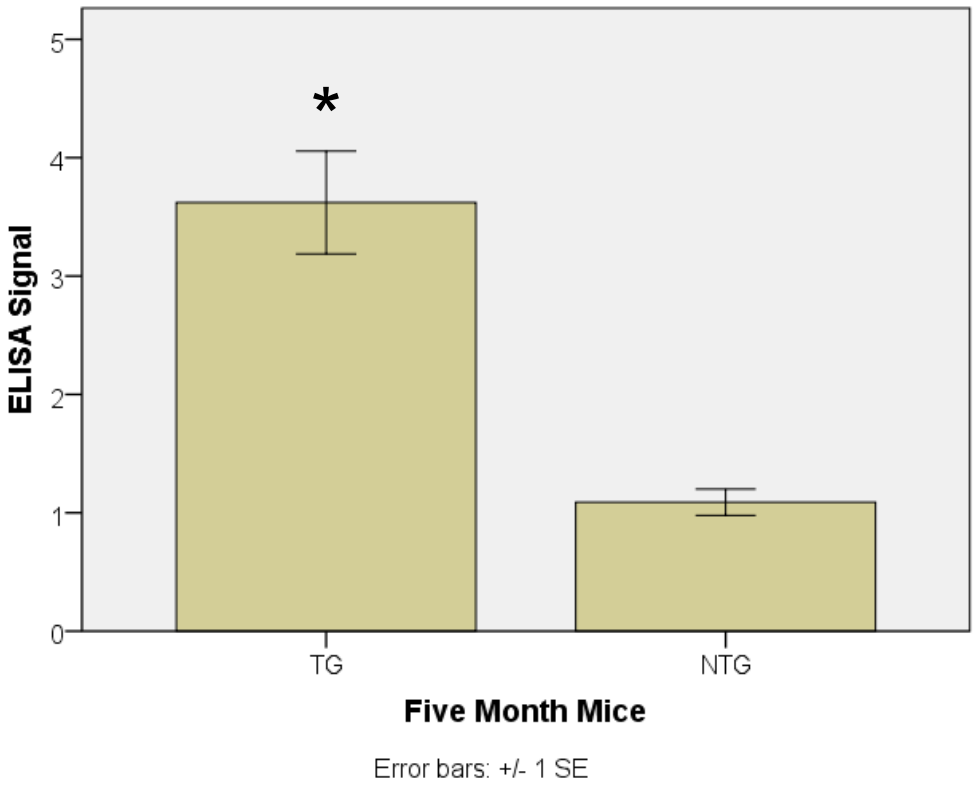

Figure 3.3. Transgenic (TG) versus nontransgenic (NTG) five month mice.

*P-value is less than 0.05 when compared to the wild type mice.

The nontransgenic and transgenic nine month mice groups each contained three mice. The data was normalized again so that the average of the nontransgenic mice group would be equal to approximately one. The nontransgenic mice obtained an average signal of 1.01 ± 0.14 where the standard error accounted for roughly 14.3% of the average. The triple-transgenic mice maintained an average signal 1.99 times larger than that of the nontransgenic mice of 2.01 ± 0.48 where the standard error was 23.8% of the average (see Figure 3.4).

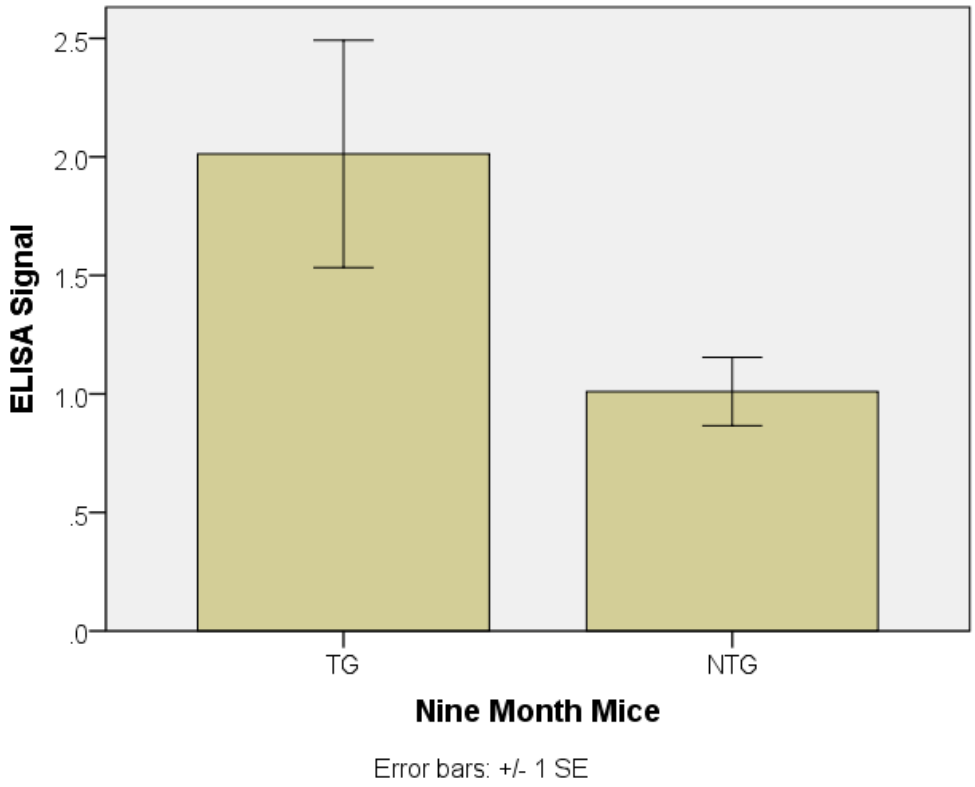

Figure 3.4. Transgenic (TG) versus nontransgenic (NTG) nine month mice.

The final mice groups tested were the transgenic and wild type thirteen month mice where each group included three mice. The data of this experiment was normalized such that the nontransgenic mice group had an average signal of roughly one. The average signals of the transgenic and nontransgenic mice groups were roughly equivalent at 1.08 ± 0.09 and 1.04 ± 0.21, respectively. While the standard error of the transgenic mice was only 8.4% of the average, the standard error of the nontransgenic mice group was a sizeable 20.6% of the 1.04 average

signal. The average signal of the transgenic mice group was a mere 1.04 times that of the nontransgenic mice (see Figure 3.5).

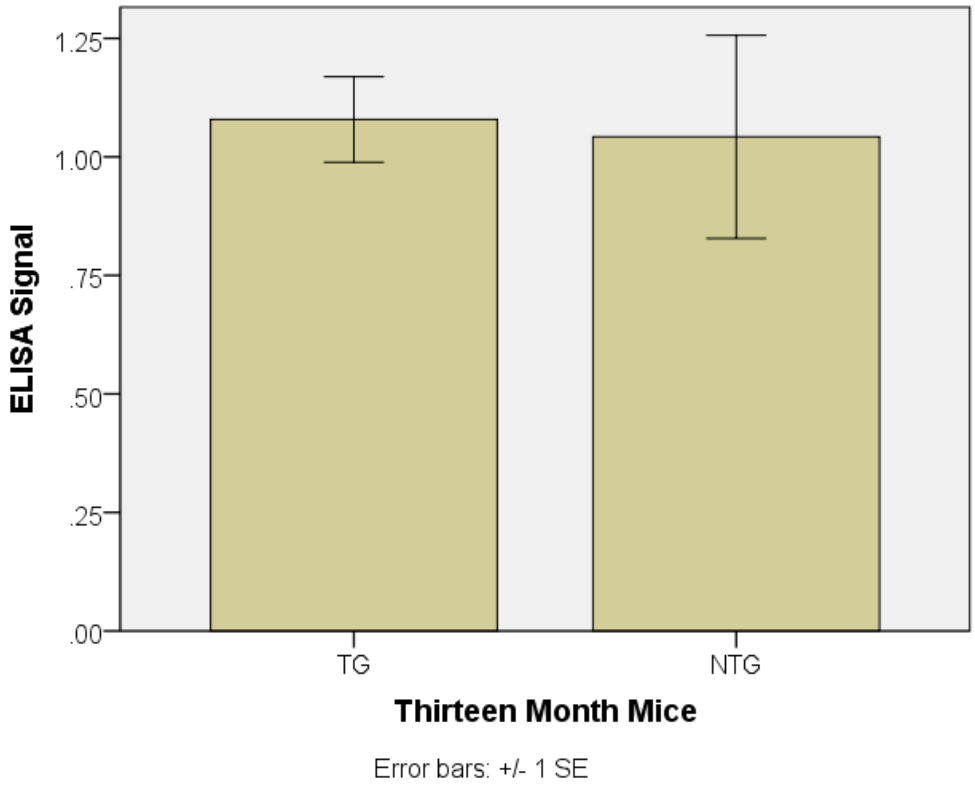

Figure 3.5. Transgenic (TG) versus nontransgenic (NTG) thirteen month mice.

The three transgenic mice groups were graphed with the three wild type mice groups in Figure 3.6. For this graph, the data was not normalized, and instead the raw ELISA signals were graphed. There is a clear decreasing pattern with increasing age for the triple-transgenic mice groups, whereas the opposite pattern is observed for the nontransgenic mice. Each transgenic mice group achieves an average ELISA signal greater than every wild type mice group (see Figure 3.6).

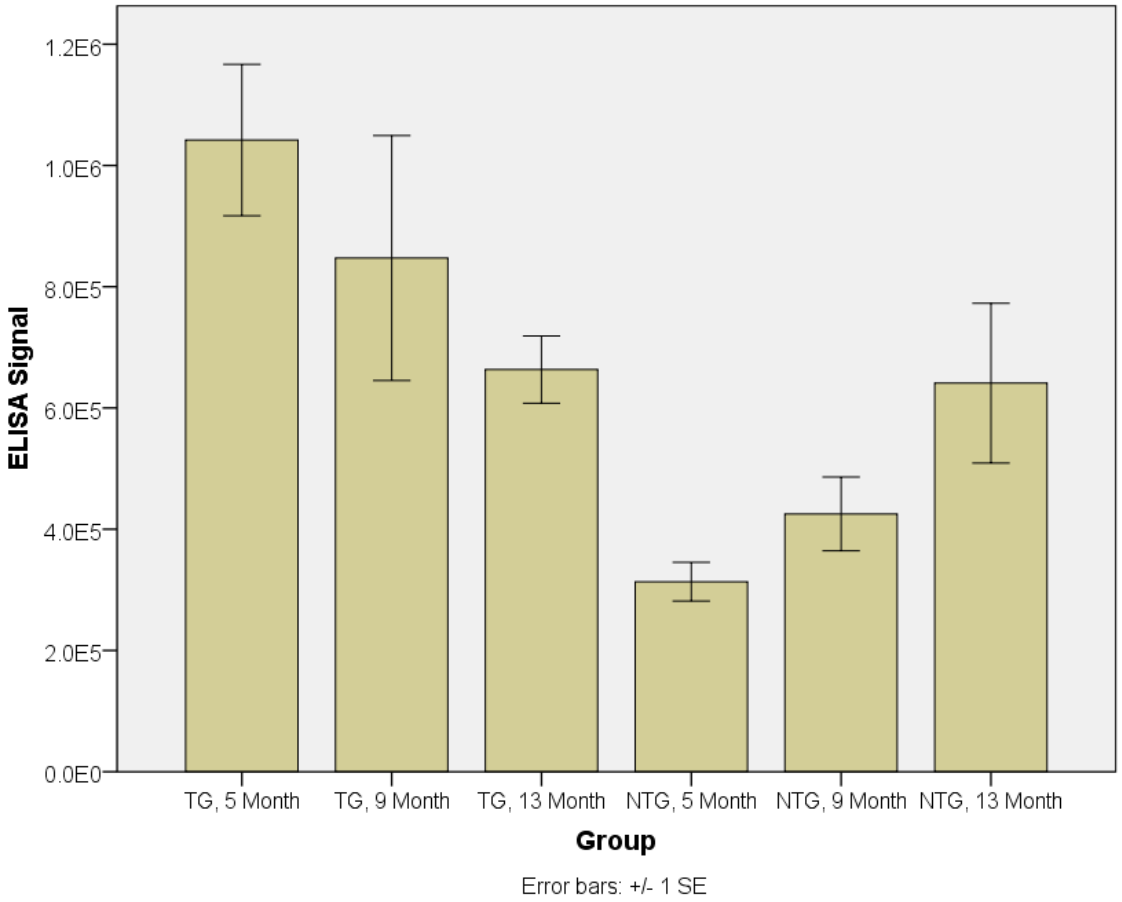

Figure 3.6. Transgenic (TG) versus nontransgenic (NTG) mice.

3.4 Discussion

The average signal of the triple-transgenic five month mice was 3.32 times higher than the corresponding nontransgenic mice group of the same age. The p-value for these mice groups was 0.000212. The p-value is more than 230 times less than the 0.05 significance level. Therefore the data presented was extremely significant, showing a defined difference between the transgenic and nontransgenic five month mice groups. The significant difference between the 3.62 ± 0.43 transgenic average and the 1.09 ± 0.11 wild type average was likely due to the abundant presence of beta-amyloid oligomers in the brain tissue of the transgenic mice (Klein et al., 2001). In the early stages of Alzheimer's disease, there is a high level of toxic beta-amyloid oligomers which trigger the neurodegeneration (Pike et al., 1993; Howlett et al., 1995; Klein et al.,

2001); however, with the progression of time, these oligomers readily form beta-amyloid fibrils (Klein et al., 2001) which are no longer detectable by the specialized A4 scFv phage capture ELISA. This graph shows that triple-transgenic five month mice had significantly higher levels of beta-amyloid oligomers present in their brain tissue when compared to five month nontransgenic mice.

The difference between the triple-transgenic and nontransgenic groups for the nine month mice was less exaggerated than that for the five month mice. This is because the average signal of the nine month transgenic group was only 1.99 times greater than that of the nine month wild type mice. A p-value of 0.063 was calculated between the two groups, 26% higher than the significance level of 0.05. At this stage in the disease, the quantity of beta-amyloid oligomers found in the triple-transgenic mice was still distinguishable from that of the nontransgenic mice because the TG average signal was larger than the NTG signal; however, there was no significance with these findings because the p-value was greater than the 0.05 significance level. A portion of the oligomers had changed into fibrils (Haass and Selkoe, 2007) such that there was no significant difference between the triple-transgenic and wild type nine month mice tissues. While the ± 23.8% error bars of the transgenic mice suggested that there may be significance, there was too much uncertainty in the data to confidently affirm significance.

There was essentially no difference between the 1.08 ± 0.09 triple-transgenic average signal and the 1.04 ± 0.21 nontransgenic average signal of the thirteen month mice. This was supported by the fact that the transgenic average was only 1.04 times that of the wild type mice group. An insignificant p-value of 0.877 was calculated for these thirteen month groups. It is likely that by thirteen months in the mice model, soluble beta-amyloid oligomers no longer exist in the brain tissue (Klein et al., 2001; Haass and Selkoe, 2007), accounting for the ELISA signal similarity between the TG and NTG groups. The beta-amyloid oligomers had collected to form beta-amyloid fibrils and could no longer instigate neurodegeneration because the pathogenesis had already occurred (Lashuel et al., 2002). If one were to consider the lower bound of the wild type error bar (0.83), and compare this value to the 1.08 ± 0.09 average signal of the transgenic

mice, the calculated p-value would still be larger than 0.05. Thus the difference in ELISA signal between the NTG and TG mice was not significant.

The clear decreasing trend of the five, nine, and thirteen month transgenic mice followed the theory that the beta-amyloid oligomers were forming beta-amyloid fibrils with time (Haass and Selkoe, 2007); however, the nontransgenic mice did not display this pattern. It could be argued that beta-amyloid oligomers were forming in the nontransgenic mice as the age of the mice increased, thereby accounting for the increasing ELISA signal with age in the wild type mice. In 1994, it was shown that there was a relative abundance of beta-amyloid peptide variants in individuals with Alzheimer's disease and those with normal aging (Näslund et al., 1994). However, there was an important difference between beta-amyloid found in triple-transgenic mice and that found in nontransgenic mice. The beta-amyloid in the TG mice was pathogenic and triggered the toxic mechanisms and metabolic pathways through the mutated proteolytic processing of APP by beta- or gamma-secretase cleavage (Holsinger et al., 2002; Hardy and Selkoe, 2002). Conversely, the amyloid protein in wild type mice was not pathogenic and was properly cleared to retain normal function (Hardy and Selkoe, 2002).

3.5 Conclusion

The average signal of the five month transgenic mice (3.62 ± 0.43) was 3.32 times greater than that of the wild type five month mice (1.09 ± 0.11). Correspondingly, the calculated p-value was 0.000212, which denotes extreme statistical significance. This was a result of the abundance of beta-amyloid oligomer proteins in the transgenic mice compared to the relatively negligible quantity of Aβ oligomers in the nontransgenic group. In the presymptomatic stages of Alzheimer's disease, there is a high level of pathogenic Aβ oligomers in the brain tissue, supporting the ELISA signal findings of the five month mice. A p-value of 0.063 was calculated for the transgenic and wild type nine month mice, revealing no statistical significance at the 0.05 level. At this stage in the neurodegenerative disease, the beta-amyloid oligomers had transformed into Aβ fibrils, reducing the ELISA signal and resulting in no statistical difference between the TG and NTG nine month mice.

A large 0.877 p-value was calculated for the 1.08 ± 0.09 transgenic average signal and the 1.04 ± 0.21 nontransgenic average signal of the thirteen month mice. After thirteen months, the plentiful beta-amyloid oligomer morphologies had completely aggregated into beta-amyloid fibrils. This morphology change caused the ELISA signals to be indistinguishable between the transgenic and wild type thirteen month mice. As the age of the transgenic mice increased, the ELISA signal decreased, graphically illustrating the aggregation of beta-amyloid oligomers with time. In stark contrast, as the age of the wild type mice increased, the ELISA signal also increased, supporting the finding that the generation of beta-amyloid oligomers also occurs with normal aging.

CHAPTER 4

CONCLUSION

4.1 Summary

A non-invasive test for the earlier detection of Alzheimer's disease is essential to prevent a 160% increase in the number of AD cases by 2050 (Bateman et al., 2012). Oligomeric beta-amyloid is a promising biomarker that can be employed for the earlier detection of Alzheimer's disease. This thesis utilized an optimized phage capture ELISA with the A4 scFv to quantify the level of beta-amyloid oligomers in homogenized triple-transgenic and nontransgenic brain samples from a five, nine, and thirteen month mouse model of AD.

The average ELISA signal for the five month transgenic mice (3.62 ± 0.43) was statistically different from the ELISA signal of the wild type five month mice ($p < 0.0005$). This was likely a result of the abundance of Aβ oligomers in the TG mice and the scarcity of oligomers in the wild type mice (Klein et al., 2001). There was no statistical significance ($p > 0.05$) between the TG nine month mice and the nine month NTG mice samples. Similarly, there was no statistical difference ($p > 0.05$) between the TG and NTG thirteen month mice. This may be due to the aggregation of beta-amyloid oligomers to form beta-amyloid fibrils (Haass and Selkoe, 2007). A clear decreasing trend with age was noted for the transgenic mice, suggesting that the beta-amyloid oligomers were accumulating to form fibril morphologies (Haass and Selkoe, 2007). The amount of oligomeric beta-amyloid in the NTG mice increased with the age, indicating the development of oligomeric beta-amyloid with normal aging (Näslund et al., 1994).

4.2 Recommendations for Future Work

Future work includes testing additional early-age homogenized mice brain samples, such as two or three month mice models. The difference between the two month or three month transgenic and wild type mice tissue samples may demonstrate even greater statistical difference than did the five month TG and NTG mice samples. Additionally, it is recommended that the phage capture ELISA be further optimized to quantify the beta-amyloid oligomers in human cerebrospinal fluid and blood plasma samples.

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