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TESTING THE AMYLOID TOXICITY HYPOTHESIS OF ALZHEIMER'S DISEASE IN TRANSGENIC *CAENORHABDITIS ELEGANS* MODEL

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1. ABSTRACT

Alzheimer's disease (AD) is affecting more people every year due to the increase in elderly population. This disease is characterized by senior plaques, containing aggregated amyloid beta peptide (A beta), and neurofibrillary tangles in the AD brains. The A beta depositions are thought to increase in cellular oxidative stress, which subsequently produces neuronal cell death in the patient's brain, causing loss of memory and, in the latter stages, dementia. Diverse models have been established to test this "Amyloid Toxicity Hypothesis of AD". Among these, the use of the nematode *Caenorhabditis elegans* has some advantages. This invertebrate has its entire genome known, as well as numerous gene homologues to those seen in humans. In relationship with the cell model, the nematode gives the benefit of an organismal view of the disease. The nematode's short life span proves useful, when compared with that of mice, allowing mechanistic studies of the disease and pharmacological treatments. Alongside with other laboratories, we have used this *in vivo* model to correlate the Aβ expression with its toxicity through the observance of the organism's behavior to provide a better understanding of the cellular processes underlining AD.

2. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder affecting the elderly population. Since the aged population worldwide has increased in number during the last years, AD has progressively gained attention to study its mechanism and consequently find a treatment, or may be even prevent its development. This is a disease affecting 10% of people over 65 years old and almost 50% of the population over 85 (1, 2). As well, there is a small portion of the population, ages between 40 and 50 years, suffering from early-ons*et al*zheimer's due to genetic disorders. This leads to a total annual national cost, direct and indirect, for its treatment and care giving of around 100 billion dollars (1, 2).

Diagnosing AD can be difficult at times since one of its most common symptoms is forgetfulness, a common characteristic of aging. In people with AD, the forgetfulness is accentuated and increases at a very fast pace, and a noticeable element is added to the personality of the individual, that of dementia. Although, CAT scans or MRI's and cognitive tests can be administered to identify AD, it is only after brain autopsy that there can be total certainty of the disease (1, 2), with the presence of Amyloid ß peptide (Aβ) deposits as the hallmark. Due to this, AD at times might go undiagnosed for several years, so by the time the doctor starts treating the patient a great deal of irreversible neuronal damage has occurred.

Aß-(1-42) along with free radicals are implicated in the major pathologies associated with AD. The Aß aggregations seen in AD brains can cause the production of more free radicals, and so the use of free radical scavengers such as vitamin E, *Ginkgo biloba*, and others may reduce these aggregation's neurotoxicity (3). Also, metals with catalytic activity to produce free radicals (iron, copper) are found in the brain of patients with AD, along with lesions caused by free radical themselves such as DNA damage, protein oxidation, lipid peroxidation, and advanced glycosylation end products (3). A better understanding of the mechanisms of neuroprotection will be important for the development of preventive strategies that target early pathological processes in age-related neurodegenerative disorders.

3. THE AMYLOID HYPOTHESIS OF AD

A characteristic AD brain contains neurofibrillary tangles, senile plaques, and synapse loss (4). The aggregation of insoluble amyloid beta peptide (Aß)-(1-42) is thought to be responsible for the formation of these plaques (5), and that of insoluble tau protein for the tangles

(6). The difference in Aß expression between late- and early-onset AD is that early-onset produces an increase amount of Aß-(1-42), while in late-onset only about 10% of the patients display this characteristic. The other 90% of late-onset AD do not have excess expression of Aß-(1-42), but they do experience deposition either because of inadequate clearance or elements that hasten Aß aggregations (7).

The primary constituents of the senile plaques are aggregates of Aβ, a 4kD peptide cleaved by β and γ secretases from the amyloid precursor protein (APP) (8). The Aβ monomers form oligomers and polymers, which assemble into protofilaments and then fibrils (9). An intriguing question is whether the Aβ fibrils or the oligomers are the cause of neuronal death in AD. The controversial theories are: 1) neurotoxicity of Aβ is directly linked to its state of aggregation in that only fibrillar Aβ is toxic (10, 11); 2) fibrils are not necessary for neurotoxicity and small aggregates or oligomers of the soluble Aβ are the neurotoxic species (12-16); and 3) generation of ROS by Aβ and subsequent protein oxidation is a possible cause for neurodegenerative diseases in AD (17-20). The evidence for or against these hypotheses is critical for determining the mechanism of Aβ toxicity and the specific therapeutic strategies.

4. THE C. ELEGANS MODEL OF AD

In order to study AD, different models have been established. Typically, the cell model provides a focused view on the specific cellular and molecular structures affected by Aß aggregation, but it fails to show the interactions between the organism's systems. The vertebrate model, mice, illustrates a more realistic analysis of the pathological interactions due to Aß aggregations, however it requires more time to obtain observations and results due to their life span. Finally the *C. elegans* model, an invertebrate with its entire known genome, has a short life span (about 20 days) and is able to express human genes in its system.

As an AD model, *C. elegans* advantages are numerous. Over half of their genome has a human homologue gene, rendering it easy to target a specific gene for study and treatment since their entire gene map has been identified. As well, these nematodes are self-fertilizing hermaphrodites (laying eggs from day three through day seven or eight) and they have proven to be helpful at evaluating drug mechanisms since their genetic and cellular features of apoptotic cell death have been described. Hence, this model not only requires less time than mice to grow and develop the disease, but it can also show the molecular interactions of the pathology of interest in a short period of time through an organismal view. Through all of these features *C. elegans* provides the prospect of a treatment for certain human diseases, in this particular case for AD, which can later be tested in the vertebrate model before human trials.

The more rare form of AD, early-onset AD, may be caused by mutations in presenilin 1 (PS-1) and presenilin 2 (PS2), or in the APP gene. Whichever mutation is responsible for the disease, they both produce an overload of Aß aggregation and oxidative stress (21). Presenilins are mainly expressed in neurons, nevertheless these have been also located in glial cells (7). Although related to the APP, PS-1 and PS-2 also have a broader role in the cellular mechanisms (7). Through the study of two *C. elegans* proteins, sel-12 and spe-4, homologues to the human presenilins, it was determined that the presenilins were involved in intracellular membrane protein passage (7). More specifically, sel-12 and spe-4 are related to protein formation and stabilization, and to intercellular signaling and cell fate, respectively (7).

The transgenic nematode *C. elegans* expressing human Aß-(1-42) was constructed by Dr. Chris Link (4, 5, 22, 23). These nematodes were injected the *unc-54*/Aß-(1-42) minigene construct in their gonads, along with a co-injection of the dominant marker *rol-6* gene (23). The transgenic progeny obtained show a non-sinusoidal movement due to the *rol-6* marker. Hence, if the Aß gene is expressed, then the nematodes rotate along their longitudinal axis (4, 5), and later become paralyzed (24). This lack of sinusoidal movement is attributable to the intracellular Aß deposits in the *C. elegans* muscles (23, 25).

Following this same construct, two strains can be identified: CL2006 and CL4176. The strain CL2006 is grown at 20^{o}C throughout their lifetime, having the Aß-(1-42) expressed at all times. In contrast, CL4176 is kept at a permissive temperature of 16^{o}C, without Aß expression (4, 22). In order for this strain to express the Aß, a temperature upshift occurs to the non-permissive temperature of 23^{o}C (4, 22). Link *et al* (22) explained how this temperature inducibility is caused by mutations in the mRNA-surveillance (*smg*) system of the *C. elegans.* In the case of CL4176, an inactivation of *smg-1* occurs, which allows for the mRNA translation of the Aß-(1-42) human transgene at the non-permissive temperature. CL4176 shows a more severe "roller" phenotype than CL2006, probably because CL2006 are already born with these Aß deposits and do not undergo the sudden build-up as CL4176 do (22).

To determine whether the human Aß is actually being expressed in the nematode, Fay *et al* (5) reported that the nematode's Aß combines at the C terminus with the specific antibodies for Aß-(1-42) after an ELISA assay. They also performed an immunoblot using the anti-ß monoclonal antibody 4G8 to establish if the Aß peptide expressed was the exact size. The Aß variant produced by the *C. elegans* migrates along the same time with the synthetic Aß-(1-42). Consequently, the nematode's Aß peptide is in fact the unmodified human Aß-(1-42) (5).

5. TESTING THE Aß TOXICITY HYPOTHESIS IN THE *C. ELEGANS* MODEL

5.1. Involvement of Small Heat Shock Protein (HSP-16) in Aß Toxicity

Fonte *et al* (26) reported that aB-crystallin (CRYAB) inhibited fibril formation of Aß-(1-42). According to Link *et al* (22) CRYAB is a stress-inducible

chaperone protein, whose activation may be in direct response to buildup of Aß in neuronal cells. The C. elegans CRYAB protein homologue, HSP-16, has been found in the same place of Aß intraneuronal aggregation in a new transgenic strain of C. elegans expressing Aß-(1-42) in neurons developed by Dr. Chris Link (22). HSP-16 is activated when the cell is exposed to a detrimental environment, and it may work to avoid protein denaturation (27, 28). This evidence points to the protective property of CRYAB, although the places where it is expressed are not equivalent to the areas presenting more acute AD pathology. Hence, Link *et al* proposed two alternatives for this noticeable difference; if CRYAB were protective, this would lead to highest CRYAB expression in brain regions with least pathologies, or because the cells expressing CRYAB early during the disease have already died (22).

5.2. Aß Fibers vs. Aß Oligomers

The necessity for two different strains (CL2006 / CL4176) expressing Aß aggregations comes from the interest in determining whether Aß depositions are the cause of neurotoxicity in the AD brain. According to Drake *et al* (7), there is no need for Aß aggregation in order to see oxidative stress. Using CL4176, they observed an increase in carbonyl formation after temperature upshift (4). Drake *et al* mentioned vitamin E, an antioxidant that besides reducing oxidative stress also inhibits cell death, does not affect the amount of Aß deposition in the brain, supporting that Aß aggregation is not needed to cause toxicity in the brain (29). They concluded that a pre-fibrilllar form of Aß exists, responsible for causing neurotoxicity. If this is true, many therapies against AD will prove useless for they are targeting the fibrillar form of Aß. The imbalance of pre-fibrillar versus fibrillar Aß caused by these therapies would produce a more toxic effect in the brain (4).

This pre-fibrillar Aß variant seems to be located intracellularly, either due to endogenous formation or to extracellular uptake (26). Along the same line, immunohistochemical analysis was performed on neurons, which lead to the conclusion that after neuronal lysis the formerly intracellular Aß becomes a factor for plaque formation (26).

Yatin *et al* (21) performed an EPR spectroscopy on Aß-(1-42) replacing a methionine (key for ß-sheet structure) near the C-terminus for a norleucine and on the reverse peptide. After this substitution, there was no increase in oxidative stress nor a decrease in neuronal survival. This showed that the ß-sheet structure of aggragated Aß is essential for its neurotoxicity. Along the same line, Fonte *et al* (26) observed the interaction of a nonamyloidic Aß variant with the HSP-16. The observation of the association between these two was not as tight as that of HSP-16 with the amyloidic Aß variant (26). Both of these independent experiments point to the fact that the conformation and structure of Aß is essential for its toxic properties to become evident.

5.3. Extracellular vs. Intracellular Aß

Previously, it was believed that the majority of Aß deposits were extracellular in AD brains, consequently the question arose of whether the *C. elegans* model was accurate in mimicking the pathology of Aß aggregation given that this nematode expresses the deposits intracellularly (24). According to Fonte *et al* (26), in *C. elegans* the Aß is not secreted from the muscle cells, even though it is recognized as an atypical protein. Instead, it is refolded and stays in the cytoplasm, although at times it may be degraded (26). However, intracellular Aß-(1-42) was detected in hippocampal neurons (24), one of the most affected areas in AD pathologies. Increasing studies suggest that the intracellular Aβ play a role in early pathology of AD. Thus, this model still seems to prove useful in studying the pathologies of AD along with any possible treatments.

5.4. Oxidative stress vs. Aß toxicity

Drake *et al* have associated Aß-(1-42) toxicity with oxidative stress using the *C. elegans*, demonstrated by the increase of protein carbonyl formation (4) in nematodes expressing human Aß. Hence, although the Aß aggregations have been identified as neurotoxic to the brain, oxidative stress has been predicted to come before these aggregations (4) causing cell apoptosis. This observation goes along with the free radical hypothesis of aging, which states that there is an imbalance of free radicals and reactive oxygen species (ROS) in the brain causing a significant damage to key cellular components (30). This imbalance may be the causative agent for the pathology of neurodegenerative disorders (such as AD) since most of these disorders are associated with older age (3).

The toxicity of free radicals was pointed out by Hensley *et al* (31) to be dependent on the kinetics of their production, as well as on their stability and transfer efficiency to lipids and proteins. For this reason, the production of free radicals by Aß deposits must be specific. There is also the probability that these radicals may interact with other radicals to produce aggregates (31). Furthermore, these radicals could promote the cleavage of the Aß precursor protein, amyloid precursor protein (APP). And so, this maintains the idea that AD can be attributed to continuous oxidative stress, along with a weakened antioxidant status (31).

The formation of free radicals by the reduction of metal ions was observed by other researchers (21). The resulting Aß was a peptide radical, since it had lost an electron in reducing the metal ion (21). Accordingly, it can be stated that Aß has distinct capacities to produce free radicals, which at the same time produce more Aß deposits. This cycle continues and causes more oxidative stress on the neurons as time goes by, provoking an increase in cell damage and death.

C. elegans have been repeatedly used to study the different AD characteristic pathologies next to its treatments in an *in vivo* model. One of these treatments is Congo Red, postulated by Lorenzo *et al* (10). The basis for its consideration as a possible treatment for AD arises from the fact that Congo Red binds to Aß, and hence it may halt amyloid deposition and probably hinder Aß-(1-42) toxicity.

C. elegans were grown with strongly stained deposits throughout their lifetime, but there was no decrease in Aß deposits or paralysis phenotype (unpublished data).

5.5. Ginkgo biloba as a potential neuroprotectant

EGb 761 is a standard *Ginkgo biloba* leaf extract used as a popular dietary supplement by the population at large in order to enhance mental focus, and by the older populace to delay the initiation of age-related loss in cognitive function. It has been shown for the past decade through *in vivo* and *in vitro* experiments in mammals and clinical studies in humans that EGb 761 shows evidence of a range of biochemical and pharmacological effects, including cognition enhancement and stress alleviation (32). Available data in human studies have established efficacy of EGb 761 in primary degenerative dementia of AD (33-35). As well, there are records supporting the view of the extract improving learning and longevity in rats (36, 37), with neuromodulatory and neuroprotective properties in certain species (38). In spite of this, there is still uncertainty as to its effects on memory in healthy humans.

Our laboratory has previously shown that EGb 761 exhibits a diverse set of cellular and neuroprotective mechanisms through the use of cell cultures, the nematode *C. elegans*, and behavioral tests in mammals. The mechanisms comprise adjusting the cellular survival machinery (38, 39), inhibition of Aβ aggregation *in vitro* (40), and amplification of an organism's oxidative stress-response (41-43).

Free radicals are produced through an oxidative process with oxygen as its key factor. Various researches have been conducted on development of neurodegenerative diseases due to the potential significance of free radical-induced oxidative damage. Smith *et al* utilized the *C. elegans* model of AD (CL2006) to establish whether endogenous Aβ 2expression augments H_2O_2-associated free radicals levels (43). The results illustrated appreciably higher reactive oxidative species (ROS) levels in an *in vivo* AD-associated model compared to a wild type or transgenic control counterparts (43). The AD-associated strains, CL2006 and CL4176, showed 2.5 and 2.6 fold increase in endogenous ROS levels than the control *C. elegans* strains, respectively (43) (Wu, unpublished). As well, continued treatment with EGb 761 significantly attenuated the induced levels of H_2O_2-related reactive oxygen species (ROS) in these *C. elegans* (39). Treatment with a portion of flavonoid components in EGb 761, kaempferol (Kaemph) or quercetin (Querc), or vitamin C (L-ascorbate), significantly decreased ROS levels when compared to untreated transgenic control nematodes (attenuation with kaempferol by 69%) (39).

Through these results the free radical theory of aging is supported (30), along with the hypothesis of the contribution of Aß ?and ROS to the pathologies of AD. As well, it clarifies an experiment of our lab in which EGb 761 extends life span in *C. elegans* (41).Wu *et al* stated that treatment with EGb 761 of the wild-type worms extended median life span to a certain extent under normal physiological conditions, but considerably raised their maximum life span under chronic oxidative test, next to increasing their resistance to oxidative stress and thermotolerance (41). These results advocate that EGb 761 enhances the natural anti-stress system of *C. elegans*, accordingly improving stress resistance and life span.

Along the same line of lessening the effects of oxidative stress, the molecular mechanism of EGb 761 was investigated using transgenic *C. elegans* expressing a jellyfish green fluorescent protein (GFP)-tagged inducible small heat-shock protein gene (*hsp-16-2) (42)*. By the use of the pro-oxidant juglone, the expression of *hsp-16-2* induced was extensively reduced by 86% in the transgenic nematode treated with EGb 761. This effect of EGb 761 correlates with its capacity to increase the mean survival rate due to acute oxidative stress, as well as to diminish the basal levels of H_2O_2 , both in the nematode (42). Subsequent effects of EGb 761 in relation to *hsp-16-2* expression (42) suggested that it functioned as a scavenger for oxidative free radicals hence preventing the dissemination of free radical damage, and also as the enhancer of repair of damaged macromolecules. For that reason, Strayer *et al* inferred that the suppression of *hsp-16-2/GFP* expression related to the ability of EGb 761 to decrease cellular stress consequential from exogenous treatments, therefore leading to a decreased transcriptional induction of the reporter transgene (42). Since small heat shock proteins are certainly expressed in Aß-expressing *C.elegans* (22), this result proposes a modulatory role of the extract in a stress-response gene role, and specifies that the effect of EGb 761 is beyond its known function as a scavenger for oxidative free radicals.

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