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Instructors:
GARRY R. BUETTNER, Ph.D.
LARRY W. OBERLEY, Ph.D.

with guest lectures from:
Drs. Freya Q . Schafer, Douglas R. Spitz, and Frederick E. Domann

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The chicken or the egg: ROS and Aβ plaques in Alzheimer's brains

by

Katrina S.M. Pedersen

Department of Radiation Oncology
Free Radical and Radiation Biology Program
The University of Iowa
Iowa City, IA 52242-1181

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Aβ Amyloid-beta protein
AD Alzheimer's disease
APP Amyloid precursor protein
ECSOD Extracellular superoxide dismutase
EPR Electron paramagnetic resonance
FAD Familial Alzheimer's disease
MnSOD Manganese superoxide dismutase
ROS Reactive oxygen species

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Abstract

Alzheimer's disease is a devastating dementia for which no exact mechanism has yet been elucidated. One of the leading theories for pathogenesis indicates that Aβ plaques frequently found in Alzheimer's brains are actually a causative agent. Researchers have noted that in regions where the plaques reside, greater levels of oxidative damage and neurotoxicity can be found. This observation has lead to the theory that ROS and other radicals may be the effector molecules inducing cell death in the hippocampus. Others, though, have noted that most ROS cannot travel across the plasma membrane and that oxidative stress may be elevated before plaques form. In order to resolve the debate surrounding the hypotheses, we have proposed research *in vitro* and *in vivo* that aims to elucidate the chronology of these events and determine the pathogenic mechanisms.

Introduction

The human nervous system is arguably the most vital system for maintaining life in that it coordinates the function of every other organ system and allows communication between the body and its environment by processing sensory information [1]. In spite of its importance, the building blocks of nervous tissue, the neurons, are postmitotic and can not be regenerated once damaged [1]. This characteristic is believed to contribute greatly to the process of aging as functional decline may be propagated through all organ systems under nervous control. Notable changes to the central nervous system include the loss of brain mass as the ventricles and sulci (fissures) enlarge and the gyri (ridges) diminish [1]. This leads to a decline in mental responsiveness with age. Also contributing to the lagging nervous response time is a decreased rate of impulse conduction along neurons. This is due to the loss of glial cells, such as the oligodendrocytes that form the myelin sheath, decreased levels of neurotransmitters and their receptors, and decreasing dendrites leading to a deficiency of synapses [1]. Interestingly, the normal aging process in the mouse brain, a common model system for the human brain, usually involves a spectrum of increased oxidative burden and antioxidant responses in various regions [2]. Also, some detectable quantities of plaques that are mainly composed of amyloid-beta protein (Aβ) are found in the normal brain in an age-dependent manner [3,4]. These changes are striking when one considers that the pathological changes to the brain during Alzheimer's disease (AD) are not unlike the alterations seen in normal aging.

The symptoms of Alzheimer's disease were first described over a century ago, and the disease is the most prevalent human dementia that is currently afflicting approximately 2% of the population in industrialized nations [5]. It is clinically

recognized by the loss of short-term memory with a subsequent loss of long-term recollection and other intellectual capabilities. Additionally, the victim may undergo behavioral changes, some of which are affected by the changing brain structure and others that are due to emotions felt by the patient, and lose their sense of time [1]. Following an extended period of neurodegeneration, the patient may experience seizures. Death is the endpoint of the disease.

Current observations estimate that, while about 5% of AD cases are heritable, 95% of all AD incidences are spontaneously initiated [4]. On a cellular and molecular level, one may note plaque formation in AD-afflicted brains. The protein amyloid-β is the main constituent of these plaques. The association between AD and Aβ accumulation in plaque formation appears to have first been made in 1990 when Yankner *et al.* noted that the direct application of Aβ protein to cultured neurons caused greater cytotoxicity than seen in controls [6]. This data was reaffirmed by Behl *et al*. in 1992 [7]. Several Aβ variants have been shown to be present in plaque species. The variants are all created from the same precursor protein (APP) differ only by the length of the mature polypeptide, such as Aβ1-40 and Aβ1-42. As mentioned above, researchers have shown that the Aβ plaque variants seen in Alzheimer's affected tissue are the same as those found in plaques within non-demented controls [3]. Surprisingly, this result shows that there is little difference between normal and disease phenotype besides the proportions in which the plaques are found. The Aβ is able to aggregate to create plaques when its precursor protein, APP, is erroneously cleaved [8]. APP is a membrane-spanning protein containing three cleavage sites, α, β, and γ, with the majority of its residues residing outside the cell. The protein undergoes two successive cleavages catalyzed by secretases,

the second of which is always at the γ site located within the membrane-spanning region of APP [8]. It may initially be alternatively spliced at either the α or β sites by its corresponding secretase. Mutations in APP have been seen in familial forms of AD, but not in spontaneous cases [3]. Recent evidence using PNA-locked PCR to detect single nucleotide variances in mitochondrial DNA (mtDNA), however, suggests that while the APP mutations seen in familial AD (FAD) are not a factor in sporadic pathogenesis, missense mutations in the control region of the mitochondrial genome may add a genetically-related component to non-familial AD pathogenesis [9]. The same researchers also employed quantitative RT-PCR to show that such mutations are expressed in AD brains and not in controls. The importance of mtDNA sequence integrity will be discussed shortly. While p3 (the α cleavage product of APP) has not been found to aggregate into plaques, Aβ is at the core of plaque formations and is seen in the memory centers of the brains, such as the hippocampus, amygdala, and entorhinal cortex [8]. Together, these data have contributed to the belief that Aβ is integral in AD pathogenesis. The amyloid-beta hypothesis states that the accumulation of Aβ in regions associated with memory causes a cascade of molecular events that eventually result in AD [5].

Many key biochemical reactions involve the transfer of electrons between molecules: one becomes oxidized (loses an electron) as a coupled molecule is reduced (gains the electron lost from the oxidized species). These reactions are particularly prevalent in the metabolic processes occurring in the mitochondria of every cell. For instance, during the process of oxidative phosphorylation in the electron transport chain, electrons enter either complex I or complex II from glycolysis and the Krebs cycle. They

are transferred from the molecules of NADH or $FADH_2$ that serve as electron acceptors between metabolic pathways to iron-sulfur complexes. Here, the complexes are oxidized and reduced sequentially in controlled reactions to build the proton gradient across the mitochondrial inner membrane [10]. The endpoint of these reactions is the formation of ATP. Because this process requires exact functioning of many various elements, it is not uncommon for electrons to leak off of the chain at complexes I, II, or III and reduce molecular oxygen before an electron pair is donated from complex IV. When a single electron reduces the oxygen, it becomes the free radical superoxide.

Reactive oxygen species (ROS) are mostly derived from the superoxide molecule and include hydrogen peroxide and its reduction product the hydroxyl radical, believed to be the most oxidizing radical in the cell [11]. As a defense mechanism, the mitochondria contain manganese superoxide dismutase (MnSOD). MnSOD can scavenge superoxide by protonating the radical and then reacting with HO_2 to convert the molecule to hydrogen peroxide [11]. The peroxide may then diffuse out of the mitochondria to the cytosol, and it may be converted to water by catalase or glutathione peroxidase [11]. In cases in which Fe^{2+} is present, however, the hydrogen peroxide may not be converted into harmless water but, rather, will participate in Fenton reactions to create the hydroxyl radical [eg. 21]. The Fenton reaction mechanism couples the oxidation of unbound Fe^{2+} to Fe3+ with the reduction of hydrogen peroxide to hydroxyl radical in any compartment of the cell [11]. Because of this chemistry, free iron in the cell may contribute indirectly to cytotoxicity if the propagated hydroxyl radicals cause enough damage to vital structures.

ROS can target essentially any cell structure. Superoxide and hydroxyl radicals have the potential to oxidize peptides, an action that can alter protein structure and ultimately their function; however, because of their reactivities, they are essentially confined to the spaces in which they were generated. This event can affect any number of biological mechanisms. Radicals may also oxidize nucleic acids and create thymine dimers, abasic sites, and base conversions. While this damage may be repaired with varying success in maintaining sequence fidelity in nuclear DNA, there are no known mechanisms to fix aberrations in mitochondrial DNA. Since mtDNA mostly encodes components of the electron transport chain, changes in the genetic code could lead to deleterious mutations in the amino acid sequences leading to structural changes that allow further unpaired electrons to leak off of the chain. The cycle continues until the cell is either no longer able to produce enough energy to sustain itself or the cytochrome c component of the chain is released, triggering apoptosis [10]. Lipid peroxidation is another result of oxidative damage. If the lipid structure is compromised after enough of this damage, the cell membrane may burst, and the cell will die [11]. Thus, it is critical for the level of prooxidants (i.e. free radicals) to be balanced by the proper antioxidant defenses to maintain homeostasis.

Oxidative damages, such as those described above, have been one proposed mechanism to satisfy the amyloid-beta hypothesis for Alzheimer's pathogenesis. The association between oxidative stress and AD was, to my knowledge, first noted in 1992 when Behl *et al.* reported that the application of Aβ directly to neurons caused cytotoxicity *in vitro* [7]. The neurons had increased levels of hydrogen peroxide, the dismutation product of MnSOD, and vitamin E, an antioxidant that could protect the cells

from prooxidant propagation [7]. Butterfield and his colleagues later demonstrated that the exogenous administration of Aβ peptides to neurons in culture lead to neurotoxicity and that lipid peroxidation was increased in those cells associated with Aβ [12]. The connection between oxidative stress and AD pathogenesis was likewise supported in the observation that, in mass spectrometric comparisons among controls, FAD, and spontaneous AD, some of those exhibiting dementia had a high degree of methionine oxidation at codon 35 in Aβ peptides [3]. Post mortem brain tissues were examined and found to have increased oxidative damage in both nuclear DNA and mtDNA [13]. Compared to age-matched controls, AD patients had a one-fold increase in nuclear DNA damage (measured by detecting OH8dG, an oxidized guanoside) while demonstrating a three-fold increase in mtDNA damage [13]. Not only does this correlate with the lack of DNA repair mechanisms in mitochondria but, more importantly, it strengthens the link between oxidative stress and AD. Taken together, these findings led many Alzheimer's researchers to investigate the role of oxidative stress in the hope of isolating a mechanism of disease induction that could be therapeutically exploited to treat disease.

The ROS Paradox

The hypothesis that Aβ aggregation induces oxidative events that eventually lead to neurotoxicity and subsequent intellectual deficits has been formed through numerous *in vitro* and *in vivo* experiments. To confirm that the presence of free radicals is in response to Aβ aggregates around cells, Hayashi *et al.* have employed electron paramagnetic resonance (EPR) to detect hydroxyl radicals in Aβ treated cells [14]. Their results reinforced the belief that hydroxyl radicals are indeed increased when Aβ is

applied to the cells. They were also able to illustrate through propidium iodide staining (though it is more an indicator of how many cell pieces exist and not necessarily cell that died apoptotic pathways) that apoptosis was more prevalent in Aβ-treated than in untreated controls [14], thereby linking Aβ, ROS, and cytotoxicity. Moreover, there is now evidence that could possibly explain how Aβ may mediate oxidative stress. In the Aβ peptide there is only one methionine residue (position 35) and, in the secondary structure of Aβ, it is in proximity with redox active metals [15]. As a thioether, the residue is readily oxidized through interactions with those metals and, upon becoming so, may be able to peroxidize lipids. This data is supported by the observation that a missense substitution of cysteine makes the protein non-oxidative and the levels of neurotoxicity have no significant difference from controls [15].

More recently, transgenic mice with knockouts in APP or antioxidant encoding genes and phenotypically selected mice have been employed to support *in vitro* results, such as those described above. Senescence accelerated mice (SAMP8) have been selected for their early onset of AD and have been used to show that knocking down Aβ expression by targeting the 42-mer region corresponding with Aβ1-42 in APP mRNA with antisense oligonucleotides (AO) significantly decrease cognitive impairments compared with those injected with non-specific AO [16]. Poon *et al*. have used this model system to investigate the effect of Aβ knockdown on oxidative species [17]. Their results show that a decrease in Aβ leads to significantly lower levels of oxidative markers (i.e. protein carbonyls, etc.). Again, this demonstrates the quantities of Aβ aggregates directly affect levels of ROS and other reactive species in AD cases. Neurobiologists have shown that in the offspring of mice overexpressing APP bred with others

heterozygous for MnSOD (+/-) had elevated levels of protein carbonyls compared to littermates homozygous for MnSOD (+/+) [18]. Additionally, the heterozygous mice exhibited significantly greater plaque burden at an earlier age, thereby suggesting the importance of being able to fully express antioxidant proteins.

While this data shows the vital role that oxidative mechanisms seem to play in AD induction, it does raise questions as to whether the Aβ causes oxidative stress or whether oxidative stress may lead to plaque burden. The fact that MnSOD is encoded on chromosome 21 and that many patients with Trisomy 21 (Down's Syndrome) have Aβ plaques much like those in AD suggests that oxidative damage could possibly lead to the disease phenotype rather than be merely the result of it. In 1999, Nunomura *et al.* using neurons from patients with AD found that oxidative damage occurs not near senile plaques as would be expected if it were producing radicals but, rather, within the bodies of susceptible neurons [19]. As mentioned above, since hydroxyl radicals are so reactive, they can diffuse only extremely short distances and cannot cross membranes. This suggests that oxidative species must be generated essentially at the site of damage, a condition that cannot be satisfied extracellularly if the radicals are formed in the cytosol [20]. Surprisingly, research has shown that oxidative damage is indirectly proportional to the level of Aβ present around and inside of neurons [19], implying that Aβ forms in response to oxidative stress and may have antioxidant properties. While metabolic abnormalities and iron release from cellular structures are required for widespread oxidation leading to neurotoxicity, it is important to remember that AD phenotypes are not fully expressed until older ages meaning that it is likely a cumulative damage leading to disease [20].

Experimental Question

As described above, much is known today about the phenotypes and the genotypes associated with Alzheimer's disease, but no one has been able to elucidate the pathogenic mechanism as yet. In terms of the oxidative stress model in support of the Aβ hypothesis for AD, the current body of data concerning the role of ROS and other reactive species has been contradictory at best. Most of the research concludes that Aβ promotes the propagation of oxidative species; however, there is growing evidence to support the proposal that, in the AD brain, ROS are elevated due likely to release of redox-active metals and perturbations in the electron transport chain leading to the accumulation of Aβ plaques, which are thought to have antioxidant properties. In our opinion, it is most likely that aspects of both scenarios may be true.

In terms of the plaques leading to oxidative stress, mouse data has shown that knocking down Aβ actually decreases ROS levels (or at least markers of oxidative damage) and fairly convincingly supports the first mechanistic order. However, it is worth noting that all of their models involved transgenic or phenotypically selected mice. Both these mice more closely model the familial form of AD because the trait is heritable and has been inbred in such animals. On the other hand, Smith *et al.* make a compelling argument for the induction of oxidative stress followed by Aβ aggregation [20]. In their case, they base much of the data from *in vitro* experiments utilizing "vulnerable neurons" obtained from AD patients of whom 95% are likely to have a sporadic form of the disease. For this reason, I hypothesize that the disease may be caused by a combined order of the two events with one or the other being favored depending on which form of the disease the patient has been afflicted with. Answering this question is vitally

important to not only the development of treatment modalities but also to the identification of environmental risk factors that can prevent AD onset before the burden of disease in many nations becomes too great.

Proposed Research

While the current state of Alzheimer's research points to ROS being associated with Aβ plaque formation, little is still known about the exact molecular mechanisms governing disease induction. One possible experiment to resolve the question of which comes first, the ROS or the plaques, would be to look at any possible redox-regulated transcription factors that may play a role in APP expression. If through promoter footprinting and other techniques we are able to see that redox-regulated transcription factor are bound to the APP promoter and that APP expression is greater following such events and later in vulnerable neurons, then we will be able to infer that ROS would have had to have been built up before the plaques were able to form. This would support the hypothesis that Aβ plaques may serve as either a sort of antioxidant or as a lesion that may create further ROS near the cell.

In order to employ methods that will help establish a timeline of possible pathogenic events, I will also initiate a longitudinal study in which I would obtain brains from sacrificed mice (with sample groups of greater than 3 in case of unrelated mortality) every month beginning at 2 months (previous data from Poon *et al.* has shown that disease arises in some mice between 4 and 12 months) allowing me to capture data from the time before disease onset [17]. I will then create two primary cell cultures from the hippocampal tissues. Since current scholarship points to some ability to regenerate

hippocampal cells, they may be able to divide in culture to a small degree. Once the neurons reach 80% confluence, I will use one culture for immunohistochemical analysis of Aβ burden at each time point using Aβ-specific primary monoclonal antibodies for detection. In order to measure levels of ROS, I could perform either DCF staining or EPR using DMPO as the spin trap in order to detect reactive oxygen species at each time point of disease progression. I would prefer to perform EPR on each sample due to the fact that the procedure measures the amount of radicals directly by stabilizing them with the DMPO molecule. By doing so, I would not have to worry as much about non-specific probe oxidation as I would in using DCF staining. Additionally, I could also obtain more accurately quantified levels of oxidative burden at each time point by using EPR with a standard curve because the numbers would not be contingent upon arbitrary gating as is required in flow cytometric methods.

If my data supports that seen in these mice by other labs, I would expect to see oxidative stress occur only after the levels of Aβ increased in the neurons. If the "ROS initiates plaques" hypothesis is supported by the data then I would expect to see increased EPR signal (or DCF signal) before noticing an increasing plaque burden with mouse hippocampus. Because it is a two-pronged experiment being applied essentially to cells from the same region I will be able to detect which is being upregulated first. Unfortunately, I know of no model system that can mimic sporadic forms of AD disease, so the results will not be able to elucidate the order of induction that can be found in the majority of AD cases. Accordingly, this methodology will not allow me to investigate more than just familial forms of disease; however, any data obtained will still support mechanistic evidence for FAD and help to either rule out oxidative stress as a causative

agent of plaque formation or confirm that stress is indeed an earlier step in disease induction than previously thought. Regrettably, I will not be able to apply this methodology to confirm the results obtained from Alzheimer's patients neurons as described by Nunomura *et al.* because it would neither be ethical nor feasible to obtain nervous tissue at controlled time points for study [19].

As mentioned in the introduction, environmental factors probably play a large role in the pathogenesis of sporadic forms of AD. Caloric restriction has been shown to increase lifespan [eg. 5], most likely because the metabolic machinery is used less thereby giving fewer opportunities in which unpaired electrons may leak off of the electron transport chain and create superoxide [11]. In the same vein, exercise increases both appetite and metabolism as more energy is required in order to sustain physical activity for periods of time. In that respect, exercise may increase oxidative damage and lead to an elevated risk in acquiring Alzheimer's disease. I intend to test this hypothesis, both in normal mice and in transgenic or senescence accelerated mice selected for dementia, by placing a wheel in the cages and following the same time course and cell culturing techniques as described above, use EPR or DCF to determine whether the ROS levels were increased any earlier in these mice compared to the earlier subjects that had no means of exercise. I will also be able to qualitatively determine whether Aβ plaque burden increased through immunohistochemical staining. I would expect, if exercise does indeed increase oxidative burden, not only would plaque burden increase earlier in both normal and dementia-selected mice populations (especially if Aβ plaques have antioxidant properties) but oxidative stress would also be elevated earlier compared to the non-exercised controls. In order to refute my hypothesis that oxidative stress increases

with environmental factors such as exercise, the data should be unchanged compared to the data obtained from the first experiment. Unlike the first study, this experiment may allow us to determine an order of ROS induction and plaque formation in spontaneous Alzheimer's pathogenesis using data obtained from the normal mice. An important secondary inference in this study that could not be obtained from the first due to the heritable forms of disease in these mouse models is that environmental factors play a significant role, even in familial disease.

In order to elucidate the order of oxidative stress induction and plaque formation *in vitro*, this time involving the possible uses of antioxidants in Alzheimer's, I will culture hippocampal cells obtained from senescence accelerated mice on a time course as described above. In this instance I shall apply purified extracellular SOD protein (ECSOD, an antioxidant similar to MnSOD but with a different activity localization) directly to the cells. If the extracellular plaques are responsible in the induction of oxidative stress, I will likely observe decreased neurotoxicity compared to untreated control cultures due to the scavenging of radicals being propagated by the plaques. If the radicals are scavenged, then there will be no source of oxidative damage to the cells and likely there will be decreased apoptosis. If elevated ROS actually causes Aβ aggregation around vulnerable neurons, then the application of SOD would either cause me to observe the lack of Aβ plaque formation if early in disease progression or would not save those neurons culled from late-term AD mouse tissues. Because ECSOD can dismute superoxide, there still may be a minimal decrease in neurotoxicity in advanced AD neurons, but it would likely not prevent apoptosis to the degree that it could if the plaques, themselves were responsible for increasing free radical burden.

Taken together, I believe that the experiments proposed above can help elucidate the contradictory data in the literature; however, until a better model system is developed, *in vivo* studies behind the pathogenesis of sporadic forms of AD will make it difficult to obtain any evidence on the causative mechanisms. While the above experiments are fairly basic, to the best of my knowledge, they have not been attempted in the literature. I believe them to be necessary in order to gain controlled results concerning the timeline of disease induction.

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