Aluminum Induced Apoptosis as a Model of Neurodegeneration

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Abstract

The fundamental mechanism for cell loss in Alzheimer's disease is currently unknown although evidence has implicated an array of contributing factors including oxidative stress and apoptosis. Recently, the potentially critical role of mitochondrial dysfunction in neurodegenerative diseases has become increasingly compelling due to the analysis of the cascade of events preceding neuronal apoptosis. Previous studies have shown that the intracisternal administration of aluminum (Al) to aged white rabbits yields many biochemical and pathological similarities to Alzheimer's disease and further implicates apoptosis. The specific aim of this project was to develop an in vitro model that uses aluminum to trigger the processes of mitochondrial dysfunction in human neuroblastoma (NT2) cells. It is hoped that this model will determine whether mitochondrial damage is a key early event in neurodegeneration. Al was shown to lead to substantial death of NT2 cells within 24 hours of incubation. Nuclear fragmentation and condensation suggestive of apoptosis was observed as early as 3 hours and increased substantially through 24 hours. Detection of cytochrome c release provided evidence that Al induced cell death occurred through apoptosis since Al treated cells exhibited reduced mitochondrial cytochrome c immunoreactivity. Taken together, these results suggest that Al induces apoptosis in NT2 cells and is subsequently a valid model to test theories of neurodegeneration as well as screen potential therapeutic agents.
Aluminum Induced Apoptosis as a Model of Neurodegeneration

Literature Review

Neurodegeneration

Every year, neurodegeneration drastically affects and often devastatingly claims the lives of countless elderly people throughout the world. The etiologies of most of the major neurodegenerative illnesses are currently unknown although most share common themes in their suggested mechanisms of cell death. While the causal mechanisms from disease to disease differ, many of the resulting pathological features are similar. Examples of the major neurodegenerative disorders included are Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and Alzheimer’s disease (AD). 7,24

Alzheimer’s disease, the focus of the current study, is fundamentally similar to PD and ALS as implicated by its neurofibrillary pathology. 24 In fact, in all three of these illnesses cumulative oxidative damage interferes with normal cellular function ultimately destroying the neurons and drastically disrupting the functional cognitive abilities of those affected. Recently, strong evidence has implicated oxidative stress as an initiator of mitochondrial injury leading to apoptosis (programmed cell death) as an instrumental mechanism of neurodegeneration in AD. Furthermore, experiments have used the intracerebral injection of aluminum (Al) maltolate into aged rabbits as an animal model of AD because of its production of observable similarities including mitochondrial dysfunction and apoptosis. 26,39,41-47 While novel in its approach, this model must be improved in order to more aptly examine the debilitating consequences of mitochondrial dysfunction and uninvited apoptosis. Therefore, a more advantageous in vitro model has
been designed in the current study which examines the effects of aluminum maltolate on cultured human neurons (NT2 cells) and subsequently provides a cleaner, less expensive, and more rapid approach than has been achieved previously.

Alzheimer’s disease (AD) was named after Alois Alzheimer in 1906 for his clinical report of a 51-year-old woman with dementia and characteristic lesions of the brain. AD is a disorder characterized by the pathological hallmarks of extracellular senile neuritic plaques (SPs), intracellular neurofibrillary tangles (NFTs), activated glia, and neuronal degeneration. This relentless, progressive dementia results in a loss of cognitive function and affects approximately 15% of adults over the age of 65 and 33% of adults over the age of 85 in Western industrialized nations.

AD is the most common of the neurodegenerative diseases with the most important risk factor being age. The study of neurodegenerative disorders, in a sense, reveals a model of normal aging where a particular age-related pathway is accelerated. For example, a small fraction of Aβ, a component of SPs, accumulates in the brains of even cognitively normal individuals. In Alzheimer’s patients, however, the rate of accumulation appears to be increased, effecting its alleged toxic neuronal degeneration. In fact, research involving aged, New Zealand white rabbits has recently demonstrated that age does indeed play a critical role in neurodegeneration, which will be explained in more detail later. The early onset of Alzheimer’s disease can be caused by mutation in the βPP (β-amyloid precursor protein), presenilin-1 or presenilin-2 genes. This autosomal-dominant inherited form usually strikes individuals by their 50s. Mutations in the βPP gene, located on chromosome 21, produce the Aβ peptide commonly found
accumulated in extracellular neuritic plaques. There are three types of mutations which occur, the most subtle being a Val to Ile mutation at residue 717, a position flanking the Aβ domain. This mutation increases the length of secreted Aβ from 40 to 42 residues, which increases the tendency of Aβ to aggregate into a polymeric form of the molecule subsequently producing extracellular neuritic plaques. Studies of human plasma, human fibroblasts, transfected cells, and transgenic mice have shown that each of these genetic forms of AD either selectively increases the extracellular concentration of Aβ42 or increases both Aβ42 and Aβ40. Thus, in hereditary forms of AD, Aβ metabolism is altered in a way that may foster Aβ aggregation and deposition. More importantly, the inherited types of Alzheimer’s disease merely account for approximately 5% of all AD cases and is consequently researched less vigorously than chronic forms of AD.

These genetic discoveries were particularly important because they showed that increased production of Aβ was sufficient to cause AD which conclusively identified βPP and Aβ as central elements in AD. However, it should be noted that while such mutations appear to be sufficient for AD, they are not necessary. This is highlighted primarily by the fact that deleterious mutations to other proteins (e.g. tau) can lead to AD and also that in virtually every published prospective study of normal aging, postmortem studies have revealed some elderly people with a form of pathological aging associated with extensive cerebral amyloid deposits who were nevertheless cognitively intact. Furthermore, since the vast majority of AD cases (95%) are sporadic and not linked to a clear genetic mutation or alteration in Aβ metabolism, research has begun to focus on uncovering a more fundamental dysfunction.
The mechanism by which chronic neurodegeneration transpires is the subject of much debate, but it is generally accepted that the release of cytochrome c into the cytosol from mitochondria leads to the activation of targeted caspases and eventual cell death. This accelerated form of AD is both chronic and progressive. Although Alzheimer’s disease has been studied extensively for the past 30 years from a variety of perspectives, recent investigation appears to be establishing a significant role for the mitochondrion in cell death, particularly including apoptosis and oxidative stress. A simplified illustration of the cascade of mitochondrial-induced apoptosis can be seen in Figure 1.

The Mitochondrion

An important topic in biomedical research related to neurodegenerative diseases involves the powerhouse of cellular life, the mitochondrion. Research has lately shown that mitochondria are responsible for the initiation of cell death. Many investigators currently speculate that neurons are particularly prone to neurodegeneration because of their requirement for great numbers of mitochondria to support their voracious energy needs. 7,24

As the major suppliers of cellular energy, mitochondria occupy a substantial portion of the cytoplasmic volume of eukaryotic cells. Mitochondrial metabolism involves the import of a freshly transformed molecule of pyruvate across its membrane and its subsequent oxidation by molecular oxygen to carbon dioxide and water. This efficient respiratory process yields 30 molecules of ATP per molecule of oxidized glucose. The usable energy produced by the mitochondria (ATP) powers the metabolic processes of the entire cell.
Figure 1. Cascade showing the key players of mitochondrial induced apoptosis.
Embedded within the inner membrane of the mitochondrion are three large enzyme complexes comprising the respiratory chain. The proton gradient established by these complexes provides enough of an electrochemical gradient to drive the production of ATP. Two mobile components carry electrons between the three major enzyme complexes, ubiquinone and cytochrome c.¹

Cytochrome c is a water-soluble peripheral membrane protein of the mitochondria. It is encoded by a nuclear gene and translated into the mitochondria where a heme group is attached covalently to form holocytochrome c.⁵⁸ Its function is to transport electrons from the coenzyme QH2-cytochrome c reductase complex to the cytochrome c oxidase complex in the electron transport chain.¹¹⁸ When released into the cytosol, cytochrome c controls the assembly of an ‘apoptosome’, a large proteinaceous complex composed of an oligomer of Apaf-1 (apoptosis activation factor) and procaspase-9 (see Figure 1). The formation of this complex results in the activation of caspase-9 and subsequent activation of other caspases leading to apoptotic death.¹⁹

Cytochrome c release has been implicated by an enormous amount of research as a key initiator of the cascade of events leading to apoptosis. Multiple, diverse cytotoxic stimuli cause the release of cytochrome c including the generation of oxidative free radicals and the loss of mitochondrial membrane potential.¹¹ This loss of membrane potential is mediated by pores found interspersed throughout the mitochondrial outer membrane called mitochondrial permeability transition pores.

Mitochondrial Permeability Transition Pore
The intermembrane space of mitochondria contains several proteins that are liberated through the outer membrane in order to participate in the degradative phase of apoptosis. \(^5\) The translocation of cytochrome c is controlled by a number of factors that control the opening and closing of the mitochondrial permeability transition pore, or MTP. Although the details are still controversial, it is generally agreed that in response to most pro-apoptotic signal transduction pathways or lethal damage pathways, mitochondrial membrane permeability is compromised, leading to the disruption of essential mitochondrial functions and to the release of soluble mitochondrial intermembrane proteins (SIMPs).

Among these potentially apoptogenic SIMPs are apoptosis inducing factor (AIF), which translocates to the nucleus where it causes chromatin condensation and large-scale DNA fragmentation; cytochrome c because it interacts with Apaf-1 to activate caspase-9; and various procaspases (2, 3 and 9) because they also participate in proteolytic destruction. \(^5\) AIF, a flavoprotein released during membrane permeability, has the ability to induce apoptotic morphological changes of the nucleus in a caspase-independent manner, whereas cytochrome c directly activates caspases by binding to a cytoplasmic protein Apaf-1 via the C-terminal WD-40 repeat domain in the presence of ATP. This oligomer complex recruits pro-caspase-9, which, in turn, induces the self-cleavage/activation of caspase-9. \(^5\) Of particular interest to the in vitro study performed here, as will be shown by the results, was the chromatin condensation and oligonucleosomal DNA fragmentation shown by TUNEL staining, and cytochrome c release which was revealed with monoclonal antibodies.
The mitochondrial inner membrane harbors a unique protein complex, referred to as the mitochondrial permeability transition pore. The MTP opens as a non-selective, high conductance 'megachannel' which allows passage of solutes of molecular weight ≤ 1.5 kD between the mitochondrial matrix and the cytoplasm. It has been confirmed that brain mitochondria undergo a permeability transition in response to many classic MTP stimuli. Opening of the MTP is controlled by membrane voltage and enhanced by a number of agents including Ca^{2+} and P_i, oxidizing agents and free radicals, free fatty acids and products of the phospholipase A2 pathway, atractyloside, and other inhibitors of the mitochondrial electron transport chain. Opening of the MTP associated with the release of Ca^{2+} currents, oxidative stress, or electron transport chain dysfunction may activate proteases and other factors leading to cell death. Hence, because of the participation of cytochrome c release in apoptosis as a direct consequence of MTP opening, investigations into the functioning of the MTP in AD models might provide insight into the mechanism of neuronal cell death.

Oxygen free radicals are implicated as important factors in signaling mechanisms leading to a number of neuropathological injuries. The role of intracellular Ca^{2+} in these signaling events is an emerging area of neurological research. Because neurodegenerative-related diseases are triggered by a number of damaging reactive oxygen species (ROS), understanding the role of oxidative stress in mitochondrial dysfunction has become increasingly popular. The reaction of a leaked electron with an oxygen molecule from the ETS will yield the superoxide radical (O_2^-) while reaction with two electrons will yield hydrogen peroxide (H_2O_2). Increases in ROS like
superoxide (O$_2^{*-}$) and hydrogen peroxide (H$_2$O$_2$), which are normally balanced by activities of superoxide dismutase (SOD) and catalase, lead to changes in cell signaling. The most devastating effect of the generation of these two relatively stable free radicals is not from their direct oxidant effects, but rather from the formation of the highly reactive hydroxyl radical (*OH), which is thought to be the most injurious aspect of oxidative stress. Because it is too short-lived to travel within the cell, depending on where it is formed, it can damage either the DNA or cytosolic and membrane-bound macromolecules. Recent studies suggest that oxidative stress may play a role in a wide range of neurological diseases. Therefore, the study of oxidative stress is important for the elucidation of membrane permeability pathways.

As stated earlier, the MTP complex is controlled by a number of agents. The most popular agents are the Bcl-2 and Bax families along with lesser known inhibitors like cyclosporin A and GDNF. Vast amounts of research and literature have been dedicated to the study of these two families of regulatory proteins. Bcl-2 belongs to a growing family of proteins which can either inhibit (Bcl-2 Bcl-X$_L$, Bcl-2w, etc.) or favor (Bax, Bcl-X$_S$, Bak, etc.) apoptosis.

The common mechanism of apoptosis is negatively regulated by several sets of genes, of which the best characterized is the ever-growing Bcl-2 family. Bcl-2 is an integral membrane protein located mainly on the outer membrane of the mitochondria, the endoplasmic reticulum, and the nuclear membrane. Bcl-2 prevents most forms of apoptotic cell death as well as certain forms of necrotic cell death and is associated with increased Bcl-2 mRNA, suggesting that increased gene transcription is responsible for the
increased Bcl-2 protein levels. Retention of cytochrome c within the intermitochondrial space is under the regulation of Bcl-2 family proteins. 11

Overexpression of Bcl-2 has been shown to prevent cells from undergoing apoptosis in response to a variety of stimuli probably by preventing the efflux of cytochrome c through the MTP complexes. 31,42 A loss in mitochondrial membrane potential (Δψ) has been correlated with the induction of apoptosis, and Bcl-2 protects mitochondria from this effect. The specific block appears to involve the MTP, which recently was confirmed to be inhibited by Bcl-2. The mechanism by which Bcl-2 actually prevents protease activation is not yet known, but current evidence leads toward a cytochrome c-activated cascade. Multiple studies incorporating a wide variety of cellular models and stressors have shown that programmed cell death can be lessened or prevented by overexpression of the anti-apoptotic proteins Bcl-2 or Bcl-XL. 6,15,37,61 This concept is supported by evidence that Bcl-2 overexpression prevents Ca^{2+} redistribution from the ER to mitochondria following growth factor withdrawal and that Bcl-2 overexpression inhibits apoptosis-associated Ca^{2+} waves and nuclear Ca^{2+} uptake. 23

These and other family-related proteins seem to function both as antioxidants 4,8 and as agents that prevent loss of mitochondrial membrane potential and subsequent release of pro-apoptotic agents such as cytochrome c. 6,15,37,55,61

Bax is a member of the Bcl-2 family, but has pro-apoptotic capabilities. Bax has been described as a death receptor, which proteolytically activates the initiator caspase-8 that in turn triggers effector caspases. 40 Translocation of cytosolic Bax to mitochondrial membranes has been associated with mitochondrial permeability alterations, release of
cytochrome c and caspase activation, which triggered apoptosis. Although Bcl-2 does not inhibit Bax translocation, it does in some cases prevent the release of cytochrome c thus inactivating the death pathways and promoting cell viability by preserving mitochondrial integrity. Figure 2 illustrates the role of Bax in the formation of MTPs and its consequential release of cytochrome c into the cytosol.

Cyclosporin A (CsA), an inhibitor of the MTP, has also been shown to prevent the early release of cytochrome c. In various situations MTP opening may be blocked by CsA, which specifically binds mitochondrial cyclophilin and prevents its interaction with the MTP complex. Other inhibitors of the MTP include ruthenium red (RuRed), which blocks the mitochondrial Ca\(^{2+}\) uniporter, ADP, Mg\(^{2+}\), and disulfide reagents.\(^1\)

**Programmed Cell Death**

The paradox presented by programmed cell death is strikingly presented by its inherent obligation to fight cancerous cells and also by its unsolicited destruction of neuronal cells during neurodegeneration. Apoptosis and necrosis are two mechanisms by which cell death occurs. The recognition of apoptosis and necrosis is based on the distinct changes that take place within the affected cells. Both methods of death involve a sequence of consecutive morphological events; however, when these two processes occur *in vivo*, they differ in their distribution and in the tissue reactions that are associated with them.

Apoptosis typically involves scattered individual cells in tissues that are removed by phagocytic cells before plasma membrane integrity is lost, thereby protecting the surrounding cells from its destructive interior contents. In contrast, necrosis involves
Mechanisms of cytochrome c release:

A  
rupture of the mitochondrial membrane

B  
a channel formed by Bax

C  
Bax and VDAC forms a novel channel

(modified from Tsujimoto and Shimizu, FEBS letters, 466 (2000) 6-10)

**Figure 2.** Bax plays an integral role in the formation of the MTP and subsequent release of cytochrome c, which binds Apaf-1 and procaspase-9 ultimately activating caspase-9 and causing apoptosis.
groups of contiguous cells that are characterized by cell swelling, chromatin flocculation, rapid loss of membrane integrity at an early phase of cell death, and eventual cell lysis. The rapid loss of membrane integrity results in the release of intracellular debris and an inflammatory response.

Apoptosis and necrosis are regulated by many of the same biochemical intermediates, most notably the levels of cellular ATP, Ca\textsuperscript{2+}, reactive oxygen species (ROS), and thiol antioxidants.\textsuperscript{36,48} Necrosis is a pathological form of cell death that gives a typical response when cells are subjected to high doses of pathological stimuli whereas at lower levels of stress the cell engages its apoptotic machinery. Whether a cell dies by apoptosis or necrosis upon injury is dictated largely by the dosage of the insult the cell receives.\textsuperscript{7,48}

Apoptosis is the essential, physiological mechanism for selective elimination of cells. It is a distinct form of cell death, in which damaged cells activate a genetic program that leads to the destruction of their DNA.\textsuperscript{22} It plays an integral part in a variety of biological events, including morphogenesis. At defined developmental periods, specific cells destroy themselves, and the cells destined for this fate can be identified as progressing along this pathway well before the process becomes irreversible. This apoptotic form of cell death occurs in neurons during development of the nervous system and may also be a prominent form of neuronal death in chronic neurodegenerative disorders such as AD.\textsuperscript{35,54} Derived from Greek terms referring to the dropping of leaves in the autumn or to male-pattern balding, apoptosis describes the morphology of cells disappearing in a noninflammatory manner.\textsuperscript{24} The term was first coined by Kerr et al.
(1972) to describe a pattern of morphological alterations associated with normal programmed cell death and certain pathological processes \textit{in vivo}.\textsuperscript{30} The typical features associated with the pathological processes of apoptosis include cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, neurite beading, chromatin condensation and DNA fragmentation.\textsuperscript{24,36,38}

There are several different apoptotic pathways, both physiological and non-physiological, and both mitochondrial and non-mitochondrial. Physiological stimuli include both external and internal signals. External physiological signals that lead to the induction of apoptosis include the withdrawal of extracellular signals as well as their appearance. Such cell death can be glucocorticoid-induced or activation-induced. Several lines of research have studied the induction of apoptosis by the withdrawal of growth factor, which causes extensive cell death, and also CTL-induced death, which kills primarily by inducing apoptosis in their targets. The most significant example of intracellular stimuli responsible for the induction of apoptosis involves the role of calcium ions, which occurs in some, but not all cell types. Inappropriate cell death has recently been attributed to increased intracellular calcium, which has been shown to be induced by aluminum interaction with calcium binding sites. Furthermore, it has been suggested that aluminum-induced neurotoxicity is an indirect effect mediated by astrocytes rather than a direct effect on neuronal cells.\textsuperscript{22}

Caspases are constitutively expressed in mammals, but different sets of caspases may be recruited in different paradigms of cell death. Non-mitochondrial apoptosis can
involve a variety of caspase activations initiated by a variety of genetic or physiological stimuli. Caspase proteases orchestrate the events that characterize the death of a cell by apoptosis. The most appropriate pathway for this study is the pathway leading to caspase activation involving the release of cytochrome c from mitochondria. Studies have shown that apoptosis-related activation of proteases is implicated in the metabolism of βPP.

Further studies showed that caspase-3, -6, -7, and -8 can cleave βPP directly. Procaspases 2, 3, and 9 are present in the intermembrane space along with AIF and their liberation is responsible for the induction of cytosolic caspases. These studies revealed that caspase activation is instrumental in the initiation of apoptosis and that the variety of caspases are responsible for different pathways.

Aluminum

Aluminum is the most abundant metal ion in the biosphere that has not yet been assigned a biological function. Although the neurotoxic effects of Al have been known for more than 100 years beginning with the pioneering studies of Siem and Dolken on experimental animals in 1897, its toxic impact on human health was demonstrated only two decades ago. Its role in certain pathologies related to long-term hemodialysis such as osteomalacia, microcytic anemia, and dialysis dementia has been well documented while the possible toxicity from cookware, food containers or other products containing Al has yet to be validated. There is little doubt, however, that Al can cause encephalopathy, bone disease, and anemia in dialysis patients. The molecular mechanisms underlying Al toxicity are not yet fully understood, in part, because of the complexity of Al chemistry in aqueous solutions at physiological pH.
The idea that aluminum has pathologically toxic effects in humans has been greatly debated for well over a century. Subtle neurocognitive and psychomotor effects and electroencephalograph abnormalities have been reported at low Al plasma levels indicating that Al is a very potent neurotoxicant. Recent studies have shown that excessive accumulation of Al the central nervous system from endogenous sources is unquestionably toxic in both experimental animals and certain human diseases including AD. A wealth of knowledge about the additional toxic effects of the metal has since been discovered in plants and aquatic animals and there are several lines of evidence that show the metal’s capacity to exacerbate oxidative events implicated in AD brain including its role as an inducer of MPT. Minute quantities injected intracerebrally into rabbits will induce severe neurological symptoms and neuropathological features of neurodegeneration.

**Al maltolate in aged white rabbits**

The development of an appropriate animal system for the study of neurodegeneration has lagged for far too long behind other avenues of investigation. The understanding of the mechanism of cell loss is hampered by the unavailability of a suitable animal model system. There is currently no animal model system available which demonstrates all of the hallmark characteristics of AD brain. However, early work with the intracerebral administration of Al compounds to rabbits has demonstrated the production of neurofibrillary degeneration. Aluminum-induced neurodegeneration in experimental animals provides a means of studying cytoskeletal changes such as are observed in some human neurodegeneration diseases. Unlike all other current animal
models, which only process individual AD characteristics, the Al maltolate-treated aged rabbit system is proving to be increasingly relevant in understanding the mechanism of neurodegeneration in AD and other neurodegenerative disorders. 39 Al maltolate is a neutral, water-soluble Al compound whose toxicity has been evaluated intravenously in male, aged New Zealand white rabbits providing an animal model to better understand neuronal cell death in AD brain. This model of AD neurodegeneration has been refined and improved over the last three decades by Dr. John Savory of the University of Virginia and has subsequently pioneered a number of worthwhile techniques and a vast array of significantly opportunistic research topics concerning the study and treatment of Alzheimer’s disease. 3,25,26,28,39,41-47

Investigations in the Savory laboratory have focused on neuronal injury resulting from intracisternal administration of the electroneutral Al maltolate complex. Rabbits are useful in studies of human disease, since their proteins show closer homology with primates than rodents. 21 Most importantly, in vivo studies with this system can address unique changes associated with aging, which predisposes the individual to neuronal injury. In fact, Savory et al. (1999) showed that in contrast to young rabbits, aged rabbits demonstrated negative Bcl-2 and strongly positive Bax associated with oxidative stress and apoptosis after 72 hours of treatment with Al maltolate, which indicated an increased vulnerability to Al-induced injury in the aged animal. 47 Histologic and immunohistochemical studies show that Al maltolate-treated aged rabbit brain resembles AD neuropathology in terms of neurofibrillary tangles, oxidative stress, and increased Aβ along with the colocalization of these with apoptosis in neuronal cells in the hippocampus
region. Table 1 compares and contrasts the characteristics of Al induced apoptosis and AD.
Table 1. Comparison of the immunopositive characteristics of Al-induced apoptosis and AD.

**Immunopositivity of AD NFTs and Al-induced NFAs**

- **Aluminum**
  - Abnormal tau +
  - P+ neurofilaments +
  - APP and AB +
  - Ubiquitin +
  - α1-antichymotrypsin +
  - α-synuclein +
  - Apoptosis +
  - Oxidative stress +
  - PHFs neg

- **Alzheimer’s disease**
  - Abnormal tau +
  - P+ neurofilaments +
  - APP and AB +
  - Ubiquitin +
  - α1-antichymotrypsin +
  - α-synuclein +
  - Apoptosis +
  - Oxidative stress +
  - PHFs +
Introduction

Because of the need for a more simplistic and accurate model to simulate the hallmark characteristics of AD, an *in vitro* experiment mimicking the rabbit model proposed by Dr. Savory was designed. The objective was to develop an *in vitro* model with which one could induce apoptosis with Al maltolate and subsequently measure the neuropathological effects as had been done previously with aged rabbits. Ultimately, human neuroblastoma cells were chosen as the most appropriate precursor cell based on its neuronal characteristics and origin. A variety of immunocytocchemical staining techniques were used to establish our model as appropriate for the testing of hallmark AD features and possible screening of future drugs.

Methods and Materials

*Cell Culture.*

Human neuroblastoma (NT2) precursor cells (Stratagene) were grown in DMEM/F-12 (Gibco) growth medium supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 1% (v/v) penicillin-streptomycin and maintained in 5% CO$_2$ at 37°C. For Al treatment, aluminum maltolate was prepared as a stock solution of 25 mM in sterile water and then filtered through 0.2 mm filter. Al was added to growth medium just before use. Unless specified, cells were allowed to adhere for 1 hour prior to replacement of media containing from 0 to 500 μM Al maltolate.

*LDH assay.*

A lactate dehydrogenase (LDH) assay was used to determine an appropriate Al maltolate dosage for the adequate acceleration of NT2 programmed cell death. NT2 cells
were grown in 96-well, round bottom plates at a density of 10,000 cells in 100 μL of media. After Al incubation, 50 μL of media was removed and analyzed for LDH activity using a cytotoxicity assay kit (Promega) according to the manufacturer’s directions. The kit uses a tetrazolium salt as a substrate that can be assayed with a spectrophotometer by measuring the absorbance at 490 nm. The percentage of viable cells is determined by subtracting the amount of LDH activity present in the media from the total LDH activity obtained from lysed cells.

**Immunocytochemistry.**

NT2 cells were grown on glass coverslips placed in 6-well plates. 1.5 x 10^5 cells in 3 mL media were plated for 24 hours prior to addition of Al. Cells were fixed in 4% formaldehyde for 15 minutes followed by a wash with PBS. Permeabilization was accomplished with an incubation in ethanol:acetic acid (19:1) for 20 minutes. Nuclei were stained with Hoescht 33258 (Sigma) at 10 μg/mL for 20 minutes. To determine cytochrome c release, an antibody to cytochrome c (Pharmingen) was used with an FITC conjugated secondary antibody. Coverslips were mounted with VectaShield (Vecta Laboratories) and the cells were observed under fluorescence with an Olympus microscope. Pictures were obtained using a digital camera and ImagePro+.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL).**

For the detection of DNA fragmentation, NT2 cells were grown on glass coverslips and placed in 6-well plates. Growth media containing 500 μM Al maltolate was added and allowed to incubate at 37°C at incremental time intervals of 3, 16, and 24 hours in 3 of the wells allowing for 3 controls. Cells were fixed in 4% formaldehyde for
15 minutes followed by 3 washes for 5 minutes with PBS. Permeabilization was achieved with an incubation in 0.2% Triton-X for 5 minutes followed by 3 washes for 5 minutes in PBS. The coverslips were then processed for apoptosis detection using an Apoptosis Detection System, Fluorescein (Promega). Positive controls were incubated with 1 unit/ml of Dnase I for 5 minutes and then continued through processing. Negative controls were refused Tdt enzyme during processing. After processing, the coverslips were mounted with Vectashield and examined with an Olympus microscope under fluorescence. Digital photographs were taken and recorded with ImagePro+.

Counts.

Apoptotic nuclei percentages were determined by obtaining images of Hoescht-stained cells from 10 random fields at 40X using an Olympus microscope, digital camera and ImagePro+. The total number of cells and the number with apoptotic morphology (strongly condensed or fragmented nuclei) were found for each image and then divided to yield a percentage for each slide.

Results

Toxicity/Viability Assay.

We examined the effect of aluminum maltolate on the NT2 cells at concentrations of 5 μM to 500 μM. Concentrations of 250 μM to 500 μM provided substantial cell death in less than 24 hours while dosages under 250 μM had minor, although significant effects (less than 20% cell death) (Figure 3). Spectrophotometric quantification of Al-treated cells was conducted at 490 nm for absorption of LDH. Because of the nearly complete depletion of cells at 500 μM Al maltolate in 24 hours, this dosage was chosen
for use throughout the study. A dose-dependent toxicity can be observed, although there appears to be a threshold at or below 250 µM of aluminum.

% Apoptotic cells (Nuclear fragmentation).

Apoptotic nuclei were recognized by typical characteristics suggestive of cell death including membrane blebbing, cell shrinkage, and irregularly multi-lobed nuclei. The percentage of apoptotic cells was estimated for control and Al-treated NT2 cells at 3-, 16-, and 24-hour incubation periods with minimal standard deviations even for Al-treated cells. Percentages of apoptotic cells with regard to nuclear fragmentation were 12.85%, 33.67% and 51.66% for Al-treated incubations at 3, 16 and 24 hours, respectively. These control and Al-treated results have been summarized in Figure 4. Photomicrographs of nuclear fragmentation shown by Hoescht staining can be seen in Figure 5.

Cytochrome c release.

The visual assessment of cytochrome c release revealed complementary results to those of the apoptotic nuclei. Release of cytochrome c appeared to be correlated with apoptotic cells implicating Al maltolate’s involvement in triggering its release. Control cells show bright fluorescence indicative of harbored cytochrome c while apoptotic nuclei were recognized by irregular shape, nuclear shrinkage and diffusely dim fluorescence. Photomicrographs of the release of cytochrome c can be seen in Figure 6.

TUNEL positivity.

Estimations made about preliminary results seem to indicate that prolonged incubations with Al maltolate corresponded with increased DNA damage as shown by
Figure 7 (A-D). The majority of TUNEL-positive cells exhibited a diffuse distribution of TUNEL-labeled nuclear chromatin. A small number of scattered TUNEL-positive nuclei exhibited a distribution of labeled chromatin suggestive of an apoptotic process, including chromatin condensation and the formation of blebs.
Figure 3. Aluminum maltolate induced significant death of NT2 cells after a 24 hour incubation period. Cell viability was determined by measuring the amount of LDH released into culture medium.
Figure 4. The percentage of apoptotic nuclei was significantly increased as early as 3 hours after incubation with aluminum maltolate. The percentage of apoptotic nuclei increased at 16 and 24 hours of incubation. In contrast, the percentage in control cultures remained fairly constant.
Figure 5. Staining with Hoescht reveals nuclear morphology of NT2 cells. (A) Control nuclei have a normal, oval appearance. In contrast, aluminum maltolate treated cultures (B) have an apoptotic morphology characterized by condensed or fragmented (arrows) nuclei. Apoptotic nuclei also tend to be brighter. Note also the decreased cell number in Al treated cultures.
Figure 6. Antibodies to cytochrome c showed a marked decrease in intensity in aluminum treated cells. This is consistent with release of cytochrome c from the mitochondria into the cytoplasm. In control cultures, cytochrome c immunoreactivity could be readily detected inside mitochondria with green fluorescence.
Figure 7 (A-D). Photomicrographs of TUNEL positivity. The Negative (A) and positive (B) controls were made for comparisons and exhibit the expected results for TUNEL staining. A small number of TUNEL positive NT2 cells can be seen in the 3-hour control (C), which is expected for cells going through their cell cycle. Many fluorescent green, condensed TUNEL positive cells can be seen in the 3-hour Al-treated NT2 cells (D). Under a higher magnification, a portion of these TUNEL positive cells are associated with nuclear fragmentation.
Discussion

The specific aim of the experiment was to test whether aluminum induced oxidative stress and triggered apoptosis as a direct effect of its action on neuronal mitochondria in vitro. Initial testing was performed on isolated rabbit brain mitochondria, but such a heterogeneous source of neuronal and glial cells possibly obscured the release of cytochrome c, which subsequently shifted our attention to human neuroblastomal (NT2) cells. This system proved to be more advantageous primarily because of the use of human cells, but it also now dealt with a homogenous population, which included the necessary cytoplasmic factors not available in our previous system.

Establishment of the efficacy of aluminum’s toxicity in vitro was accomplished by counting the percentages of viable NT2 cells after incremental dosages of Al maltolate were administered. A lactate dehydrogenase (LDH) assay was utilized because of the release of the enzyme during and just before cell death. The threshold observed between 50 µM and 250 µM indicates where the neuron’s limited capability to neutralize oxidative insults approximately occurs. Although an Al maltolate concentration of 500 µM was chosen as an appropriate dosage for the acceleration of programmed cell death, it remains to be determined whether the preliminary results of TUNEL staining might indicate that the oxidative insult is too strong. Recall that a rapid, excessive insult affords a quicker, necrotic type of cell death causing rapid cell lysis consequently producing different effects than those produced here. Accordingly, future experiments will be conducted at lower concentrations of Al maltolate that are still within the threshold.
Regardless of the possible excessive concentration, the results of the various experiments performed established A1 as an effective inductor of cell death *in vitro*. Validation of the specific pathway of cell death is very difficult given the fact that the mechanisms by which apoptosis works may not be so ubiquitous. Various indicators of apoptosis like Bax/Bcl-2 changes and cytochrome c release may only involve one of the many converging pathways leading to eventual caspase activation and cell death.

Since aging has been reported to be a contributing factor for neurodegeneration, elucidating the effects of time on cellular dysfunction has led researchers to believe that an exhausted electron transport chain in the mitochondria produces ROS capable of causing membrane permeability. However, recent TUNEL experiments have shown that the treatment of animals with glial cell neuronal derived factor (GDNF) inhibits Bcl-2/Bax changes and abolishes caspase-3 activity, but does so without blocking the release of cytochrome c. This indicates that membrane permeability is not directly involved in the prevention of caspase activation, and that there are more undiscovered steps between cytochrome c release and caspase activation. Based on this new information, GDNF has been proposed as a possible therapeutic strategy to reverse apoptosis. To our knowledge, GDNF has not yet been tested as an inhibitor of caspase activation in NT2 cells and could represent a future experiment for our system. Mitochondrial membrane polarity would also be relevant to examine the neuroprotective effect of GDNF, and this can be accomplished using CMXRos. This technique would, of course, necessitate the assessment of cytochrome c release, bax/bcl-2 translocation, caspase activation, DNA fragmentation and various other apoptotic tests.
Because necrosis has been shown to elicit the release of cytochrome c along with apoptosis, supplementary characteristics should be assessed for confirmation of the use of apoptotic machinery during cell death. This idea applies to one of the original questions of the study, that is, whether Al causes oxidative stress. There are a variety of tests for the assessment of oxidative stress including monitoring of the up-regulation of proliferating cell nuclear antigen (PCNA), the fragmentation of DNA, which produces O8-DHG, and heme-oxygenase (HO-1).

Oxygen radicals are produced in all cells either by the normal cellular metabolism or by the exposure to external mutagens. The reactive oxygen species (ROS) generated can then induce DNA damage. Among the principal lesions produced in DNA by ROS is an oxidized form of guanine (O8-DHG). The importance of this lesion resides in its mutagenic potential when present in the template strand during DNA replication. The detection of this lesion is possible with a specific monoclonal antibody. O8-DHG detection indicates the possible implication of oxidative stress and subsequently the possible implication of an apoptotic type of cell death.

Mammalian DNA contains approximately 3 X 10^9 base pairs, which must be replicated accurately if the organism is to survive without cancer, or to reproduce offspring without debilitating genetic defects. DNA repair during replication is accomplished through the use of common enzymes, including DNA polymerases, DNA primase, RPA, PCNA (proliferating cell nuclear antigen), and RFC. Thus, DNA repair enzymes are targeted to sites where DNA replication creates mistakes (or encounters unrepaired damage), and DNA replication enzymes are targeted to sites of
environmentally or metabolically induced DNA damage. PCNA is synthesized during the late G1-early S phase of the cell cycle, immediately preceding the onset of DNA synthesis. It is a trimer, which functions as a sliding clamp that transforms polymerase δ into a highly processive enzyme.\textsuperscript{9,33,32} Because of its close relation to the cell cycle, PCNA is used as a physiological or pathological marker protein of proliferating cells and can be used for the detection of DNA fragmentation with the use of an antibody.\textsuperscript{27,59} Accordingly, PCNA should be expressed in cells immediately after oxidative stress causes DNA damage. Preliminary experiments were conducted with PCNA during this study, but because of the lack of repeated success, the results were not included. Further work with any of the replication enzymes is anticipated for future experiments.

After the usefulness of the precursor cells in our model has been confirmed through comprehensive testing, further experiments that would be valuable for the confirmation of the hypothesis would involve the differentiation the neuroblastomal (NT2) cells using retinoic acid (RA). The NT2 cell line, derived from a human teratocarcinoma, exhibits properties indicative of a committed neuronal precursor stage. NT2 cells can be induced by RA to differentiate \textit{in vitro} into postmitotic central nervous system (CNS) neurons, hNT neurons. All current and future tests performed on the precursor model could then be performed on the differentiated hNT neurons further substantiating the hypothesis on a model that even more closely resembles the normal human system.

The etiology and mechanism by which apoptosis occurs is still under much debate. The elucidation of such a complex system will not resolve easily and is markedly
realized when drugs like GDNF inhibit caspase activation, but fail to inhibit the release of cytochrome c from mitochondria. The challenge remains to experiment with more drugs to elucidate more pathways, and ultimately, of course, perhaps discover a ubiquitous, fundamental pathway that will afford the opportunity for treatment.

Conclusion

In summary, the purpose of the experiment was to investigate the degenerative role of aluminum *in vitro* on human neuroblastoma cells. To do this, specific characteristics of oxidative stress and apoptosis using Al maltolate on cultured human neuroblastoma (NT2) cells were identified. Three lines of evidence demonstrated the effects of Al on the cultured cells. Primarily, the dose-dependent effects of Al toxicity on NT2 cell viability was demonstrated, which gave a satisfactory concentration for use in further experiments. Immunocytochemical staining with monoclonal antibodies revealed the release of cytochrome c from the intermembrane space of the mitochondria, which demonstrated the involvement of Al in mitochondrial membrane permeability and possible oxidative stress. Oxidative stress was further implicated by the exposure of nuclear fragmentation with the aid of the nuclear stain, Hoescht. Lastly, confirmation of DNA fragmentation was accomplished using the fluorescent TUNEL staining technique. The significance of this model lies in its ability to elucidate new key players in the intricately complex machinery of apoptosis and also to test and monitor new drugs designed to counter the effects of neurodegeneration.

The hypothesis that mitochondrial dysfunction plays a central role in apoptotic cell death and Alzheimer’s disease has been demonstrated by a substantial amount of
evidence from animal models and now presently in an *in vitro* model. The current study establishes aluminum as a useful trigger for the induction of apoptosis and subsequent examination of early events.
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