HUMAN EVIDENCE IN ALZHEIMER
PRELIMINARY REPORT

ON

THE STUDIES PERFORMED

WITH
Introduction: The general concept of a systemic pathophysiological response to cerebral damage was first developed almost 150 years ago (1). Currently there are several cellular and molecular responses to acute cerebral injury have been identified. While some of the cellular responses to cerebral ischemia occur with in 15 sec. In animal models (2), increased catabolic hormone release probably occurs within a few minutes, while the cardiovascular response such as changes in blood pressure (BP) occur in 20 min. (3) However, other responses such as the cerebral inflammatory responses only begin after 20-24 hrs (4). While a response to injury may be construed as a healing process, these responses to the injury need not always be beneficial, in some instances they may add to faster deterioration of neuronal functions if the cells are not pre-primed with defensive mechanisms. Thus prevention of these deleterious effects caused after the injury or the preventive measures that reduce the damage caused by injury will have very useful clinical application. One of the preventive measures is to administer medication that enhances the protective/defensive mechanisms of the cells.

Neuronal degeneration need not always be a consequence of physical accident but can also be consequence of aging process. Aging is associated with cognitive impairments and manifestation of neurodegenerative diseases like Alzheimer’s (AD) (5) and Parkinson (PD) (6). The biochemical and molecular changes that accompany the aging process are currently being identified in various laboratories around the globe with the intention of either elimination or retardation of the age induced cognitive impairments. Some mutations in certain genes such as Werner’s disease may add to faster aging process, mutations in certain genes does not take place for aging to occur. Aging is a natural process which involves changes in gene expression. Thus regulation of gene expression is one of the ways to counter the age associated disabilities. Regulation of these biochemical and molecular changes that take place during aging should, in theory, automatically retard or even reverse the cognitive impairments thus reducing the chances of becoming victims of age associated diseases like AD and PD.

In our laboratory we have shown that the expression of several gene products in the memory centers such as hippocampus, amygdale and septum of mouse brain is altered with aging Fig 1 shows one method of analyzing the changes in gene expression during aging process. In this method we have used a technic called Differential Display Reverse Transcriptase Polymerase Chain Reaction (DDRT – PCR) to locate the changes in gene expression in the hippocampus of 4 and 12 months old mice. The arrows indicate the over or under expression of certain genes during aging process.

In addition to this technology, recent technology of micro array analysis can also be effectively used to identify the age induced changes in gene expression. In this system an array of genes is fixed into a slide or nylon paper and the mRNAs from the brain tissues of different age groups is used to hybridize with the fixed array of genes. An example of such analysis done in our laboratory is given in figure 2 (>). For this experiment, an array of toxicology and stress genes is purchased and the mRNA from 4 and 12 months old mice evas converted to cDNA and radioactively labeled. The labeled product was used to hybridize the with the genetic elements fixed on the array. The arrows show the changes in gene expression during the aging process. These technologies can be virtually used for any type of scenario such as a disease process, injury etc. to identify the changes in cellular metabolic events related to the changes in gene expression.

In the case of aging, in the memory centers of the brain, among the protein whose expression changes, most notable are the amyloid precursor protein (APP), nitric oxide synthase, desaturases of saturated fatty acids, and the chaperonins (a class of stress proteins).
APP is an integral membrane protein of almost all the cells. Aberrant processing of brain APP that releases the small molecular weight insoluble fragment of A\(\beta\) deposits leads to initial loss of memory consequently causing the disruption of neuronal communication and eventual death and lysis of the neurons. The study of metabolism of APP and its processing can be done in human immortalized cell cultures of any organ in the body or in the neuronal cell cultures. Accumulation of amyloid beta protein (A\(\beta\)) which manifests as plaques in several parts of the brain is one of the events that is associated with pathogenesis of Alzheimer’s disease (AD) (8-10). A\(\beta\) is a 40-43 amino acid small molecular weight peptide which is derived by the intramembranous proteolytic processing of amyloid precursor protein (APP) by secretases (11,12). Mutation in APP has been shown to cause increased accumulation of A\(\beta\) causing AD in humans and AD like symptoms in transgenic mice (13). In our laboratory we have recently shown that it is possible to reverse the age induced cognitive defects of the senescence accelerated mice (14) by regulating the expression of amyloid precursor protein (Kumar et al 200 – submitted ; patent pending).

Similarly we also have shown in our laboratory that desaturases of fatty acids have reduced expression during aging (15). Modulation of neuronal nitric oxide also occurs during aging process (Kumar et al, unpublished).

Biochemical studies using the tissue culture experiments :

Our preliminary experiments with the estimation of APP and desaturases showed that this drug may potentially regulate amyloid processing and also increase the desaturase levels. Expression of both these proteins is affected during aging. APP increases with age and desaturase decreases with age. The drug seem to reduce APP expression and increase desaturase expression which is beneficial if occurs in human cells. In an invitro study using HeLa cells transfected with mouse APP, we have found that at concentrations ranging from 1µg/ml to 500µg/ml, there is a significant reduction in APP expression. Interestingly, a reduction of APP expression appears to take place even at 1 µg/ml. These experiments will have to be repeated to establish the dose and time dependent changes in APP expression.

**Experiments with the animals :**

As mice are closest to man in mimicking the changes, these experiments will help to organize clinical trials in humans. Treatment of the 12 month old SAMP8 mice with this drug for three weeks by oral feeding in their water was performed. These animals are currently being tested for their acquisition and retention abilities. The SAMP8 mice(derived from their parental AKR J strain) exhibit memory deficits at 12 months of age. Therefore, these mice serve as excellent model for the study of memory deficits and their improvement with this drug.

These three proteins and some of the proteins presented in DDRT – PCR and microarray are conserved evolutionarily and can be modulated by molecular methods.
Experimental results by using the supplied Dr. Rajkumar:

**Effect on tissue culture cells:**

Human neuroblastoma cells were grown in tissue culture and physical changes in the neurons in the presence and absence of the drug were studied. For this various concentrations of the drug was added to the neuronal cells and 48 hours after the addition of the drug, cells were analyzed microscopically, Figures 3 shows the control neuroblastoma cells 48 hours after seeding.

The cells were treated with different concentrations of for 48 hrs. Fig. 4 shows the morphological changes in neuronal cells with just 1 µg/ml of the drug. Even at this low concentration cellular elongation and induction of neuronal filament formation is clear.

Increased amounts of the drug has more interesting effect. Fig. 5 and 6 show the addition of 100 and 200 µg/ml of the drug respectively. These two concentrations have exhibited two properties. Firstly there is an increased amount of neuronal filament formation it. Every viable cell in the culture two concentrations. Secondly, the cell number has increased by 30% in the presence of the drug and the cells appear to be more healthy. Further, higher concentrations of the drug did not show additional morphological changes. In fact 500 µg/ml has shown some cellular shrinkage and even some cell death. This figure is not enclosed. Prolonged incubation with 500 µg/ml did cause recovery of the cells and eventual filament formation. The filament formation is essential for cell – cell contact and is probably related to increased memory. While the physiological mechanism of memory is not well understood, it is assumed that cell contact is an essential requirement for increase in memory and further retention.

In addition to the concentration curve we have also performed the time course of induction of filaments in these cells in culture. These data are not enclosed with this preliminary report. In general we have noticed that physical changes in the cells in cultured takes place in less than 6 hours after the addition of the drug and appear to peak out by 48 hours.

The morphological changes that are noticed in the neuronal cells must be accompanied by metabolic changes. The two methods of identification of changes presented in the introduction of this report are universally applicable. We intend to perform the DDRT – PCT and / or micro arrays to investigate if some of the vital gene expression changes we observed during aging are reversed by this drug. Secondly, we also intend to perform the experiments that estimate the levels of APP desaturase and nitric oxide in the neuroblastoma cells before and after treatment with the drug. Further, experiments with animals is crucial for obtaining convincing evidence for physiological changes occur in mammals also. This would pave the way for clinical trials.
The animals were tested for their retention and acquisition capabilities after 3 week continuous feeding of
the drug. Fig. 7 A shows the loss of acquisition abilities with age in these mice. By 12 months, these mice show a
high retardation in their cognitive faculties. When they are treated with the drug for three weeks they fully
recover to normal state of their counter parts (the R1 mice) and behave like a 12 month old strain. The neuronal
changes in the brain by in situ staining and the levels of APP, desaturase and NOS both at transcriptional and
transnational level need to be performed using the typical Western blot and RNase protections assays.

**Human Studies:**

Once the animal studies are completed, we will be able to design human studies for clinical trails. As the drug is
obtained from natural sources, the obstacles to set up these experiments will be minimal. As there is already
documented evidence that the drug functions well in humans in other countries, in USA the study can start as
phase 2 or phase 3 clinical trial, particularly because of the available tissue culture and animal data.

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