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USE OF GENETICALLY ENGINEERED MICE AS MODELS FOR EXPLORING THE ROLE OF OXIDATIVE STRESS IN NEURODEGENERATIVE DISEASES

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1. ABSTRACT

A growing body of evidence has suggested that oxidative stress may play a major role in the degeneration of neurons associated with several neurological diseases of aging including ALS, Parkinson's, and Alzheimer's disease; this has been the topic of numerous previous reviews and opinion papers (e.g. 1-10). The ability to construct genetically engineered mouse lines containing targeted mutations has done much to aid in the assessment of the role of reactive oxygen species (ROS) in both the initiation as well as the progression of these diseases and has markedly advanced research in the field. Most importantly, the creation of genetic animal models has strengthened the argument that antioxidants may be a useful therapy in the treatment of these types of disorders.

2. INTRODUCTION

Until recently, the only animal models available to the neurobiologist for studying age-related neurodegenerative diseases in which genetics were believed to play either a primary or predisposing role were spontaneous genetic mutants. In the last two decades, however, techniques have been established for the introduction of selected mutations *in vivo* as a means of mimicking human disorders. The ability to create laboratory mouse strains containing targeted mutations has helped extend our understanding of many diseases including several of the neurodegenerative diseases associated with aging. Genetically engineered mouse strains have given us important information concerning various factors involved in degeneration of the nervous system during normal aging and in age-related disease states and have provided valuable animal models for testing new drug treatments.

Ectopic expression of novel genes can be achieved through a process called transgenics which involves the microinjection of cloned DNA into the pronuclei of fertilized mouse eggs (11). Depending on the site of chromosomal integration, the DNA can be transcribed and translated into a functional protein. The injected eggs are then introduced into pseudopregnant females and allowed to develop. If integration into the mouse's genomic DNA occurs at the one cell stage, the integrated DNA will be contained in all cells of the mouse's body including its germ line cells and therefore becomes a heritable complement of its genetic make-up. Normally, the DNA construct used for transgenic production is designed to contain the gene of interest expressed under the control of a regulatory promoter element which designates when (i.e. at what developmental times) and where (i.e. in

what cell types) transgene expression will occur in the genetically engineered mouse.

A second type of genetic engineering called gene targeting (also called "gene knock-out") can be used to alter endogenous genes of interest in the mouse's DNA (12-13). First, the cloned gene fragment is altered *in vitro* often via insertion of a neomycin (neo) resistance gene into an exonic coding region of the gene. In addition, thymine kinase sequences from the herpes simplex virus (HSV-tk) are introduced at both ends of the linearized transgene. The altered gene is then introduced into pluripotent embryo-derived stem (ES) cells in culture by either direct injection or electroporation. Homologous recombination occurs between the altered transgene and the endogenous ES gene at the region of homology between the transgene and endogenous genomic target sequence. During homologous recombination the distal HSV-tk sequences are eliminated. Cells containing the homologously integrated copy of the gene can therefore then be selected by growth in media containing neomycin and gancyclovir. The selected cells are then introduced into mouse blastocysts where they can become any part of the tissue of the developing animal including the germ line cells. Resulting chimeric offspring are bred resulting in mice which, if the ES cells have become part of the germ line, carry the alteration in one copy of the gene in all cells (ie heterozygous mutants). These can be further bred to obtain mice in which the mutation is found in both copies of the gene (ie homozygous mutants).

These techniques have been used to create a myriad of genetically engineered animal models, some of which have been extremely important in allowing scientists to examine the role of oxidative stress in a number of neurodegenerative disease states. A number of these studies are described below.

3. DISCUSSION

3.1 Amyotrophic Lateral Sclerosis

ALS is a progressive neurological disease manifesting during middle age and resulting in degeneration of motor neurons of the brain stem and spinal chord, subsequent muscle weakness, paralysis, and eventually death (14). Although most cases of ALS are sporadic (SALS), approximately 5 to 10% of ALS is familial and is mostly inherited in an autosomal dominant fashion with varying degrees of penetrance (FALS).

3.1.2 Mutations in SOD are involved in some forms of ALS

A subset of FALS (about 20%) as well as some forms of SALS have been demonstrated to result from mutations in the Cu/Zn superoxide dismutase (SOD) gene (15-18). SOD is a 154 amino acid metalloprotein involved in the enzymatic conversion of superoxide anion to hydrogen peroxide in the cytoplasm; another form of the protein, Mn SOD, performs the same function in the mitochondria (19). Hydrogen peroxide can be further detoxified to water by either catalase or glutathione/glutathione peroxidase.

Exactly how mutations in the SOD gene can result in the specific disease pathology observed in ALS is difficult to assess in humans due to the lack of homogeneous genetic backgrounds and environmental conditions in patients with the disease. Therefore, in order to determine the mechanism by which these various ALS SOD mutations elicit their damaging effects on motor neurons, mouse lines containing some of the same mutations found in human ALS were created.

3.1.3 Studies on genetically engineered mice containing human ALS-SOD mutations

Three distinct transgenic ALS mouse models were created in different laboratories by the introduction of a human SOD gene containing one of the mutations found in familial ALS patients, i.e. change of a glycine to arginine at position 86, glycine to alanine at position 93, and glycine to arginine at position 37 (20-22). High levels of mutant gene expression in the central nervous system of these lines correlated with development of symptoms similar to those observed in ALS patients, i.e. degeneration of motor neurons, muscle atrophy and weakness, paralysis, and death by middle age (23).

Importantly, the expression of mutant SOD proteins in the genetically engineered mouse lines did not, in every case, result in a loss or decrease in the ability of SOD to scavenge superoxide radicals. In fact, the degree of motor neuron degeneration in these animals was found to be better correlated with actual levels of the mutant enzyme itself rather than its effects on superoxide levels (24). Furthermore, deletion of endogenous Cu/Zn SOD in another genetically engineered mouse line did not result in motor neuron degeneration implying that the neurodegenerative effects elicited by the mutant SOD gene product is likely to arise due to gain of an aberrant function by the SOD protein rather than loss of its normal activity (25).

It has been suggested that the toxicity of the mutant SOD protein may be due to its inability to bind and sequester free copper which is found at the active site of the enzyme and is a necessary cofactor for its activity (26). Free copper would then be available to react with hydrogen peroxide produced during breakdown of superoxide by SOD to produce highly reactive hydroxyl radicals (OH.) which are known to cause cellular damage to nearby proteins, nucleic acids, and membrane phospholipids. However, a comparison of enzymatic activities of the purified Gly to Ala mutant SOD enzyme vs. wild-type demonstrated that both contain the same number of bound copper molecules at their active sites (27). Free radical generation, however, does appear to be enhanced in at least one of the mutant ALS-SODs due to a decrease in the Km for hydrogen peroxide as substrate for the enzyme. This has been postulated to be caused by increased accessibility of the mutant enzyme for hydrogen peroxide due to imperfect folding of the enzyme protein resulting in an increase in the size of the active channel. The increased generation of .OH from hydrogen peroxide could, in turn, be responsible for the observed motor neuron degeneration (28). Another possible explanation is that the increased size of the active channel allows increased use of peroxynitrite as substrate resulting in the formation of highly reactive nitronium intermediate which can then react with free tyrosine or tyrosine on protein residues (29). Neither of these theories

explain, however, why motor neurons show particular susceptibility.

3.1.4 Free radical production in mutant ALS SOD-containing mouse lines

To test whether the the effects of the ALS SOD mutations were due to either increased .OH production from hydrogen peroxide or increased tyrosine nitration via peroxynitrite, levels of both were measured in two lines of transgenic mice expressing FALS mutant forms of the SOD enzyme (30). While there was no evidence of an increase in hydroxyl radical formation, levels of 3-nitrotyrosine were found to be elevated by 2-3 fold in the spinal chord in both of the ALS SOD lines throughout the course of the disease. The presence of nitrotyrosine demonstrates that tyrosine nitration is an aberrant *in vivo* property of at least some of the ALS SOD mutations.

It is not clear from these studies whether there are specific proteins which are targets for tyrosine nitration, but neurofilament proteins might be likely candidates for such modification. Both SALS and FALS are characterized by an abnormal accumulation of neurofilaments in motor neuron axons suggesting that neurofilment accumulation may play a role in the disease (31-33). In addition, increased neurofilament production in transgenic animals overexpressing the neurofilament heavy chain gene results in disorganization of neurofilaments, impediment of axonal transport, and subsequent motor neuron degeneration. Motor neurons may be particularly susceptible to neurofilament abnormalities due to their high rate of synthesis of neurofilament proteins. However, in the ALS-SOD mouse mutant lines examined, no increase in tyrosine nitration of neurofilament proteins was detected (30).

As peroxynitrite is normally formed in the cell via reaction of nitric oxide (NO.) with superoxide, it would be interesting to examine the effects of crossing the ALS-SOD mutants with various available transgenic lines which are deficient in the NO synthase (NOS) enzyme (34). In addition, although results from some of the earlier studies described above seem to indicate that peroxidation of hydrogen peroxide is not involved in neurodegeneration in at least the particular ALS-SOD strains examined, it would still be informative to look at the effects of crossing novel ALS SOD mutant lines with available genetically engineered lines expressing elevated or decreased brain levels of cellular glutathione peroxidase (GSHPx, 35) to see if this alters the course of the disease.

3.2 Parkinson's Disease

Parkinson's disease (PD) involves a selective loss of dopaminergic neurons in an area of the midbrain called the substantia nigra (SN). Loss of these neurons results in decreased dopamine (DA) production in regions of the brain which are innervated by these cells including the striatum (ST), nucleus accumbens, cortex, and thalamus (36, 37). The circuitry made up by these various connections is involved in modulating voluntary movement and loss of appropriate signaling in this circuitry results in deficiencies in motor function characterized by muscle rigidity, jerky movements, rhythmic resting tremors and both akinesia and bradykinesia, i.e. the inability to initiate or to complete voluntary motor movements. PD is relatively prevalent in the aged population, occuring in one out of every 100 individuals over 65 years of age and results in a progressive neurodegeneration which ends in death primarily due to secondary complications such as infections (7).

Dopaminergic neurons are believed to be particularly prone to oxidative damage because they contain DA which can undergo auto-oxidation or be oxidized enzymatically by monoamine oxidase (MAO), the principle enzyme in the body for the catabolism of intracellular catecholamines (38). Oxidative deamination of dopamine produces reactive oxygen species (ROS) such as hydrogen peroxide which can react with free iron to produce OH. radicals which can react in turn with cellular components which could eventually lead to degeneration of dopaminergic (DAergic) neurons. The presence of regionally high levels of both iron and MAO in the area of the SN is believed to contribute to the susceptibility of this region to free-radical damage.

3.2.1 MPTP toxicity as a model for PD

In mice, the systemic administration of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in changes reminiscent of those seen in PD including decreases in striatal DA and its metabolites, tyrosine hydroxylase activity, and in both dopamine binding and uptake, all indicators of a loss in DAergic nerve terminals (39-41). Mice interperitoneally injected with MPTP also display a marked loss of DAergic cell bodies in the SN similar to that observed in PD.

The compound is believed to cross the blood-brain barrier following injection and to be converted by the B isoform of MAO to its active form, 1-methyl-4-phenyl pyridium (MPP^+). MPP^+ is then taken up by a receptor-mediated process into DA neurons of the SN and once inside these cells it inhibits complex I of the mitochondrial electron transport chain resulting in a decrease in ATP synthesis and leading to cellular degeneration.

3.2.2 Use of genetically engineered mice to explore the role of ROS in MPTP toxicity

Upon blockade of complex I by MPTP, superoxide levels can become elevated and may result in increased cellular toxicity. To examine the role of superoxide radical production in MPTP toxicity, the effects of the toxin were compared in transgenic mice expressing three-fold higher levels of SOD than normal with non-transgenic littermates with normal SOD levels. The SOD transgenics, unlike the non-transgenics, demonstrated no significant decrease in levels of DA or its metabolites or in DA binding or uptake (42). This suggests that some of the deleterious effects of MPTP are through production of superoxide radicals. MPTP is similar in structure to the herbicide paraquat which has, in fact, been shown to increase superoxide production *in vivo* (43).

Increased superoxide production following inhibition of complex I activity by MPTP may exert its damaging effects either directly or indirectly by interacting

with hydrogen peroxide via the iron-catalyzed Haber-Weiss reaction to yield highly reactive and toxic OH. radicals. It has, in fact, been shown that mice which are deficient in the hydrogen peroxide-scavenging enzyme glutathione peroxidase are more susceptible to MPTP toxicity (Flint Beal, personal communication).

In contrast to the results reported above, primary DAergic neurons isolated from SOD overexpressing transgenics have been reported to show no increase in resistance to MPP+ (44). They do however appear to survive better both in culture and following grafting into rodent brain and therefore may prove useful in transplantation studies in animal models of PD (45). In addition these data suggest that antioxidants may aid in survival of transplanted fetal tissue into the brains of PD patients where free radicals generated during tissue preparation or transplant may compromise the survival of developing DAergic neurons. This could have profound effects on improving or ameliorating of symptoms in patients with this disorder.

Transgenic animals, in which brain levels of the MPTP-converting enzyme MAO-B are elevated three to four-fold above normal, do not show an increased sensitivity to MPTP (46). These results appear to imply that the rate-limiting step in MPTP toxicity in the mouse is not conversion of MPTP to MPP^+ by MAO-B but perhaps one of the other steps in the toxicity process such as transport or long-term sequestering of MPP^+ in the mitochondria or levels of MAO-B activity in brain microvessels which may act as a sort of chemical blood-brain barrier to obstruct MPTP entry into the brain (47-48). Mice which are deficient in MAO-B expression show increased sensitivity for the toxin (49).

Interestingly, by middle age, mice with elevated brain MAO-B levels display a marked decrease in cellular area in those neurons which contain substrate for the enzyme, e.g. DA neurons of the SN (50). MAO-B is known to increase with age in the brain and this has been postulated to contribute to neuronal degeneration observed during normal aging through increased hydrogen peroxide production as a by-product of the enzymatic breakdown of dopamine (51). Further *in vitro* studies suggest that increases in MAO-B levels in dopamine-containing cells results in increased ROS production and free radical damage which could account for the cellular atrophy *in vivo* (52).

3.2.3 Glutathione may act to protect dopaminergic neurons against damage mediated by energy impairment: a possible factor in PD

PD has been reported to be accompanied not only by a decrease in energy metabolism (as exemplified by decreases in mitochondrial complex I activity and increased frequency of mitochondrial DNA deletions in the striatum) but also by lowered glutathione (GSH) levels in the SN (2, 9, 53-55). Hydroperoxides are formed during oxygen reduction by the mitochondria and can have deleterious effects on tissues due to their ability to react with free iron to produce highly toxic hydroxyl radicals. The GSH/GSHPx system is the primary defense mechanism for peroxide removal from the brain. Alterations in its availability could prevent protection against formation of ROS, thereby accelerating cellular degeneration. GSH is probably particularly crucial in protecting the mitochondria against oxidative damage as this is the primary source of its generation and if functionally compromised due to ROS-generated damage can result in a further increase in ROS production. Since the brain is highly dependent on the mitochondria for its necessary energy supply, this can have severe effects on neuronal cells. It is not clear whether mitochondrial damage or oxidative stress represent the initiating event in PD, however the two are clearly linked and both are likely to contribute to the pathology of the disease.

Systemic administration of buthionine sulfoxamine (BSO), an inhibitor of *de novo* GSH synthesis, to mice results in morphological effects on the cells of the SN which are reminiscent of those reported to occur in the rodent brain with advancing age including reduction in DA content, increased lipofuscin deposition, and increased numbers of dystrophic axons in nigrostriatal fiber tracts (56). Interestingly, the neurodegenerative effects of BSO treatment on DAergic SN neurons were not generalized to other neuronal cell populations in the brain. These data suggest that these neurons may be especially susceptible to the effects of GSH reduction especially if energy metabolism is also compromised.

Transgenics with decreased brain levels of GSHPx demonstrate significantly more loss of striatal DA following intrastriatal injection of the mitochondrial toxin malonate, a competitive inhibitor of succinate dehydrogenase (Flint Beal, personal communication). This suggests that the glutathione system may play an important role in protecting DAergic neurons against the detrimental effects of energy impairment and that antioxidant treatment may slow the progression of PD.

3.3 Alzheimer's disease

Alzheimer's disease (AD) is an age-related dementia which is characterized by the increased presence of neurofibrillary tangles and amyloid plaques in the brains of affected individuals. The formation of these plaques and tangles appears to result in synaptic loss and gliosis particularly in the cerebral cortex and the hippocampus which is associated with the cognitive decline that defines the disease.

AD is the most prevalent cause of progressive intellectual failure in older individuals, characterized by severe memory loss, disorientation, and profound personality changes. Roughly 10-33% of all AD cases are believed to be inherited and several genes either causative for the disease or which act as risk factors have already been cloned (57).

3.3.1 Beta-amyloid and AD

Amyloid plaques are the primary pathological feature of the AD brain (58). A major component of these plaques is a hydrophobic 40-42 proteolytic amino acid fragment of the membrane-associated amyloid precursor protein (APP) called beta-amyloid.

A causative role for beta-amyloid in the neuropathology associated with AD is supported by several lines of evidence. In the brains of AD patients, amyloid plaques containing beta-

amyloid are surrounded by dystrophic neurons and areas of gliosis (58). Some cases of familial AD have been demonstrated to be due to mutations in the APP gene and these mutations appear to promote increased production of beta-amyloid (59). In addition, transgenic mice expressing mutant forms of APP show a brain region specific increase in beta-amyloid deposition which increases with age and results in AD-like neurodegeneration in those brain areas containing beta-amyloid (60-61). However, it should be noted that beta-amyloid plaques have also been observed in some brain areas showing no degeneration in neurologically normal individuals and synaptic loss is seen in other brain areas where no apparent beta-amyloid deposition occurs (62). These data suggest that while mechanisms other than those mediated by beta-amyloid deposition are probably involved in AD, beta-amyloid is likely to play a pivotal role in the pathogenic process.

3.3.2 ROS production as a causative agent in beta-amyloid induced neurotoxicity

Beta-amyloid causes an accumulation of hydrogen peroxide in neuronal cell lines which, in turn, results in lipid peroxidation and cell death; this process can be prevented by treatment with antioxidants (63-64). These data suggest that beta-amyloid mediates its toxic effects via ROS-induced cell damage. Exposure of cultured hippocampal neurons to beta-amyloid also results in neurodegeneration which seems to be mediated via ROS generation. Incubation of dispersed hippocampal cultures in beta-amyloid (1-40) results in an increase in ROS production along with increases in protein oxidation and lipid peroxidation which appear to ultimately result in decreased viability of hippocampal neurons (65-67). In organotypic hippocampal cultures which, unlike dissociated hippocampal neurons, maintain the synaptic circuitry of the hippocampus and therefore may be a superior model for studying the effects of beta-amyloid *in vivo*, beta-amyloid toxicity is preventable using a general synthetic scavenger of superoxide/hydrogen peroxide (68). Some recent reports from studies of the effects of beta-amyloid on primary hippocampal cultures have suggested that Aß-induced ROS may result in production of 4-hydroxynonenal (HNE), an aldehyde product of membrane lipid peroxidation which in turn can conjugate with specific proteins in the cell and in this manner elicit a detrimental effect on cellular function (67). Other recent studies, however, suggest that ROS may not be directly involved in beta-amyloid toxicity (69).

3.3.3 Studies on genetically engineered mice with elevated Aß to investigate whether ROS is a component in its neurodegenerative effects

As mentioned previously, transgenic mice have recently become available which have increased expression of a mutant form of the human APP; by 6 months, these animals develop amyloid deposits, neuritic plaques, synaptic loss, gliosis, and memory deficits similar to those seen in AD (60, 61). They do not, however, develop the neuritic tangles which are present in the brains of a majority of AD patients. The pathology acquired by these animals demonstrates that the presence of amyloid plaques alone is sufficient to cause much of the disease pathology. These animal models will enable scientists to watch the progression of the disease and to ascertain whether and at what point the build-up of plaques leads to particular AD symptoms. These animals are also likely to be important models for use in drug testing. The study of these animals might also be quite informative for delineating the role of oxidative stress in the course of the disease i.e. by looking for increases in indicators of ROS production in these animals as well as testing whether crossing these lines with other lines expressing e.g. increased or decreased levels of brain glutathione peroxidase results in the slowing or acceleration, respectively, of the observed neuropathological or behavioral symptoms.

3.4 Huntington's disease

Huntington's disease (HD) is an autosomal dominant disease characterized by disturbances in movement (ie "choreic" or jerky movements) and progressive dementia. This disease is caused by the expansion of a CAG trinucleotide repeat in the coding region of a gene on chromosome 4 encoding huntingtin, a protein of unknown function (70). The mutation results in the age-related degeneration of a subset of GABAergic neurons in the basal ganglia. Studies in mice in which the gene has been rendered non-functional via gene knockout indicate that the HD is not due to a lack of normal huntingtin function (these animals are embryonic lethals) but is likely due to a gain of function by the protein which is lethal for these GABAergic neurons (71-72).

3.4.1 Excitotoxic model of HD

Interstriatal injection of glutamate agonists such quinolate results in degenerative effects that mimic several of those present in the brains of HD patients including sparing of striatal aspiny neurons of the striatum (73-74). There is no morphological evidence of excitotoxic neuronal damage or elevated quinolate levels in HD brains, however, it has been proposed that the disease may be due to a pre-existing metabolic defect which renders cells of the striatum more vulnerable to normal physiological levels of glutamate or related endogenous compounds, i.e. "slow excitotoxicity" (75-76). In fact, a substantial amount of evidence indicates that the gene defect in HD results in impaired mitochondrial energy metabolism including decreased glucose utilization and increased cortical lactate production in the basal ganglia made as cells switch from mitochondrial respiration to glycolysis as the primary source of ATP synthesis (5).

Administration of 3-nitroproprionic acid (3-NP), an inhibitor of mitochondrial respiration, results in clinical symptoms similar to those observed in HD (77-78). It produces selective lesions in the striatum which appear to involve secondary oxidative stress following loss of ATP. Its toxicity also appears to be age-dependent. Because of the specificity of its toxicity, its systemic injection has been extensively used as an animal model of HD.

3.4.2 Use of genetically engineered mice to assess the part that ROS production may play in excitotoxic cell death

Transgenics expressing either elevated brain levels of either Cu/Zn SOD or lowered levels of GSHPx show attenuated or increased striatal lesions, respectively, in comparison to non-transgenic controls following intrastriatal 3-NP injection as well as decreases in accompanying ROS production (79, personal communication).

Transgenics are now available which contain the same mutation as that found in HD patients and show much of the same pathology (80-81). It would be interesting to examine these animals for increases in indicators of excitotoxic injury and ROS production; these animals could also be crossed with lines with increased or decreased brain SOD or glutathione peroxidase expression to see if this alters the progression of the pathology of the disease. It is intriguing that clinical trials in HD patients show that vitamin E may slow the rate of motor decline early in the course of the disease (82).

The role of oxidative stress has been extensively studied in ALS, AD, and PD, but its role in HD has not been as thoroughly investigated and use of such animal models as described above might be the first step towards understanding whether and in what manner ROS is involved in this disease state.

4. CONCLUSION

Irrespective of the primary causative events, it is likely that ROS generation plays a major role in the progression of neurodegenerative diseases and may, in fact, represent a common converging pathway in the development of neuronal cell death. Genetically engineered animals have been and will continue to be an extremely important component in the on-going analysis in this field of research.

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