Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease resulting in degeneration of lower motor neurons in the brainstem and spinal cord, leading to generalized weakness and muscle atrophy followed by death (1). Five to ten percent of ALS cases are familial and are inherited in an autosomal dominant manner. The mechanisms leading to degeneration of motor neurons in familial and sporadic ALS are as yet unclear. Motor neuron loss is accompanied by reactive gliosis, intracytoplasmic neurofilament abnormalities, axonal spheroids and axonal degeneration.

A major discovery was the finding that missense mutations in the enzyme copper/zinc superoxide dismutase (SOD1) are associated with 15-20% of familial ALS (FALS) cases (2). This has focused attention on how these mutations may result in selective death of motor neurons. The superoxide dismutases are a family of enzymes which play a crucial role in the protection against oxygen radical-induced cellular damage. SOD1 is a soluble homodimer of 153 amino acids, which is localized in the...
cytoplasm and the nucleus of many eukaryotic cells. Within each monomer there is an active site containing one ion each of copper and zinc. The major function of the enzyme is the dismutation of the superoxide ion to form hydrogen peroxide. The resulting hydrogen peroxide is then converted to water by either glutathione peroxidase or catalase. It was initially demonstrated that the mutations resulted in a shorter half-life of the enzyme as well as reduced activity \textit{in vivo} (3,4). Subsequent studies however have argued against a loss of function. Arguments against a loss of function include the dominant inheritance of the disorder, a poor correlation between loss of enzyme activity and the clinical progression of the illness, and the finding that certain mutations resulting in minimal loss of enzyme activity nevertheless result in the illness (1).

Other evidence arguing against a loss of function comes from studies in transgenic mice. It was demonstrated that SOD1 knockout mice live to adulthood and do not develop motor neuron disease (5). Furthermore transgenic mice expressing the G93A or G37R human SOD1 mutations with elevated levels of SOD1 activity, and mice expressing G85R mutant SOD1 with protein levels and activity levels essentially equal to endogenous levels, develop progressive hind limb weakness and muscle wasting (6-8). Mice which express native human SOD1 at comparable levels do not develop motor neuron disease. This evidence taken together strongly favors the proposal that the motor neuron degeneration arises from a gain of a novel property of the enzyme which results in cell injury.

Two proposals have been advanced suggesting a disease mechanism. The first is that the mutant SOD1 has an altered substrate affinity which leads to the generation of toxic reaction products and the second is that the mutant enzyme may be unstably folded so that it precipitates to form toxic cytoplasmic aggregates. It has been proposed that the mutations tend to occur in the protein backbone of the enzyme thereby leading to altered accessibility of the active site copper to a variety of substrates (9). In particular it has been proposed that there may be more ready access to hydrogen peroxide or peroxynitrite (10). Peroxynitrite is formed by the spontaneous reaction of superoxide with nitric oxide. It can be converted by the catalytic copper of the mutant enzyme into a highly reactive nitronium intermediate which then can nitrate tyrosine residues on proteins. It has been recently demonstrated that the mutant enzymes bind zinc less tightly (11,12). When zinc is not present in the mutant enzyme, the protein backbone is destabilized resulting in an increased generation of reactive nitronium ions (11), which can result in nitration of the light chain of neurofilaments. This has been shown to disrupt the ability of neurofilaments to form the normal neurofilament triplet (11), which in turn could directly contribute to the cytoskeletal abnormalities shown to be a pathologic hallmark of ALS. In support of an increase in nitration we found increased levels of 3-nitrotyrosine in transgenic mice with either the G37R or the G93A mutations (13,14). In the G37R mice, increases occur as early as 6 weeks of age. We also found increased concentrations of 3-nitrotyrosine in the spinal cord in both sporadic ALS patients and in FALS patients with the A4V mutation (15).

Another potentially toxic interaction of the mutant SOD1 molecule is with hydrogen peroxide. It was demonstrated that hydrogen peroxide can more readily react with the mutant enzyme generating hydroxyl radicals \textit{in vitro} (16). Expression of mutant SOD1 in PC12 cells is associated with increased superoxide production, and cell death is attenuated by copper chelators, Bcl-2, glutathione, vitamin E and inhibitors of caspases. Furthermore, it has been demonstrated that expression of the mutant enzyme, but not the wild type SOD1, results in increased apoptosis within cultured cells (16,17).
Another possible toxic mechanism is that the mutant enzymes are unstable and cause a release of Cu$^{2+}$ into the cell cytoplasm. This could then result in Fenton-type chemistry by reacting with H$_2$O$_2$ to generate hydroxyl radicals. Recent work showed that mutant SOD1 isolated from red blood cells shows abnormal copper peaks (18). Expression of mutant SOD1 with 2 bp deletion associated with FALS results in marked instability of the protein, increased susceptibility to proteolysis and possible release of copper. Copper levels in the brain of one patient with the 2 bp SOD1 mutation showed a two-fold increase. The neuropathology in this family is associated with intracytoplasmic hyaline inclusions in astrocytes. Copper binding and reactivity, however, were normal in isolated SOD1 with the Asp 90 Ala mutation (19). A strong argument against an involvement of copper comes from studies in which mice with a knockout of a copper chaperone are crossed with transgenic ALS mice. This markedly reduces SOD activity but had no effect on disease phenotype.

The formation of intracellular aggregates of SOD1 is another possible toxic mechanism. The expression of SOD1 cDNAs with mutations found in association with FALS led to the formation of cytoplasmic aggregates in cultures of spinal motor neurons (20). The aggregates were not observed following expression of wild-type SOD1 and they were not observed in dorsal-root ganglion or hippocampal neurons, which are resistant to degeneration in ALS. The formation of the aggregates was closely linked to apoptotic cell death (20). The formation of cytoplasmic inclusions in astrocytes in G85R transgenic ALS mice is a prominent pathologic feature (6). Supporting the possibility that SOD 1 aggregates may lead to neuronal degeneration is the discovery of neuronal SOD1 positive inclusions in some FALS patients with the A4V mutation. Intracytoplasmic inclusions have also been observed in G93A transgenic ALS mice. These findings raise the possibility that the protein aggregates may be exerting a toxic effect as has recently been postulated in diseases associated with polyglutamine expansions. Recent work has shown that protein aggregates occur early before onset of symptoms or pathology in transgenic ALS mice (21).

There have, thus far, been only a limited number of studies of therapeutic interventions in transgenic ALS mice. Most of these have been performed in G93A mice. It has been shown that both riluzole and gabapentin can significantly prolong survival in these mice (22). It has recently been demonstrated that riluzole also preserves motor function in G93A mice (23); vitamin E, meanwhile, appears to delay the onset of the illness in G93A mice but has no effect on disease course or survival (22). Carboxyfullerenes, which are free radical scavengers, can significantly prolong survival by 9 days in G93A mice (24). Treatment with penicillamine increases their survival by 10 days (25). We have been interested in the concept that agents, which may ameliorate bioenergetic dysfunction may be useful in the treatment of neurodegenerative diseases. We carried out studies utilizing coenzyme Q10 and more recently creatine in several animal models of neurodegenerative diseases. We found a small but significant improvement with coenzyme Q10, and with creatine an improvement (of around 20%) in survival (26). Creatine also delayed motor weakness and protected against loss of motor neurons.

**MITOCHONDRIAL AND CELLULAR PATHOLOGY OF TRANSGENIC MICE**

Neuropathologic studies of transgenic ALS mice have demonstrated that vacuolization of mitochondria appears to be an early and prominent pathologic feature. Both the G93A
and G37R mutants display abundant vacuoles derived from dilated mitochondria (7,8). In contrast, mice with G86R and G85R mutants do not show prominent vacuolization (6). G93A mice develop neurofilament accumulation in axonal steroids. G85R and G86R mice show a very rapid progression of the disease as well as Lewy body like inclusions in SOD1 aggregates in both astrocytes and motor neurons (6). In addition, G85R mice show a decrease in the glutamate transporter located on astrocytes (6).

A number of studies have examined the time course of neuronal degeneration in G93A transgenic ALS mice. The onset of clinical illness as assessed by fine shaking of the limbs is at ~90 days of age followed by paralysis and death at ~140 days of age. The earliest pathologic changes are vacuolization of spinal motor neurons, which are seen in mice at 37 days of age initially in proximal axons and then in the cell body at 45 days of age. By 70 days of age they are seen in most motor neurons. A significant loss of motor neurons is not seen until 90 days of age coinciding with the onset of clinical symptoms. At the end stage, there is a ~50% loss of motor neurons in all spinal cord segments. A recent study correlated mitochondrial vacuolization with motor weakness. It was shown that there is a marked increase in mitochondrial vacuolization, which immediately precedes a rapid phase of onset of motor weakness. This finding suggests that mitochondrial vacuolization is directly linked to the onset of clinical weakness in these mice and plays a pathogenetic role. Expression of the G93A SOD1 mutation \textit{in vitro} causes a loss of mitochondrial membrane potential as well as elevation of cytosolic calcium concentrations (27). A link between SOD and mitochondrial dysfunction is based on the finding that both normal and mutant SOD1 translate into mitochondria and that this correlates with vacuolization and cell dysfunction (28).

MITOCHONDRIAL-OXIDATIVE INTERACTIONS

Mitochondria are both important targets and important sources of reactive species. Because of the potential for univalent transfer of electrons from the electron transport chain to oxygen (electron leak), mitochondria are likely to be the major source of reactive species in eukaryotes (29). The generation of reactive oxidants, including reactive oxygen species (ROS), appears to be increased in damaged mitochondria and in cells with compromised mitochondrial function. Conversely, mitochondrial structure and function are extremely sensitive to oxidants. For example, as described in more detail below, acute exposure to relatively high levels of oxidants, especially in the presence of calcium, can induce the mitochondrial permeability transition, uncouple oxidative phosphorylation, and contribute to cytotoxicity via necrosis and/or apoptosis (through release of cytochrome c). The chemical composition of the mitochondria and its constituents contributes to its oxidant sensitivity. Mitochondrial membranes are highly polyunsaturated, making them excellent targets for peroxidation. Iron-sulfur proteins (e.g., aconitase, rhodanese) are abundant, essential, and highly susceptible to oxidant-mediated damage. The potential combination of free iron or copper and hydrogen peroxide from the dismutation of O$_2^-$ increases the odds of production of $\cdot$OH through Fenton chemistry. Potential byproducts of $\cdot$OH-mediated change are indeed observed. These damage byproducts include the aromatic hydroxylation products o- and m-tyrosine, which are seen in some but not all mitochondrial preparations and the DNA damage product 8-hydroxydeoxyguanosine, which may reach concentration ~15 times that found in nuclear DNA. We found significant increases in 8-hydroxydeoxyguanosine in urine, plasma and CSF of ALS patients that correlates with disease progression (30).
MITOCHONDRIAL PERMEABILITY TRANSITION

A central role for mitochondrial permeability transition (MPT) in both necrotic and apoptotic cell death is starting to emerge (31,32). MPT is attributed to a voltage-gated, cation-permeable channel, whose opening is favored by a number of factors including depolarization, intramatrix Ca2+ and oxidizing agents, and whose closing is favored by protons (low matrix pH) and adenine nucleotides. Oxidation of pyridine nucleotides favors pore opening while reduction favors pore closing. Cyclosporin A is a good blocker of the channel that appears to require an interaction with mitochondrial cyclophilin. The channel functions in both high- and low-conductance modes. Induction involves the calcium-mediated opening of a pore in the inner mitochondrial membrane that allows free diffusion of all solutes smaller than 1500 daltons in and out of the mitochondrial matrix. Induction, therefore, leads to loss of the proton gradient and inability to conduct oxidative phosphorylation.

Exposure of mitochondria to supraphysiological levels of calcium, particularly in the presence of inorganic phosphate or oxidants, leads to mitochondrial dysfunction. At least two partially interactive and partially independent biochemical mechanisms mediate this loss of mitochondrial function (31,32). The first of these is termed calcium cycling; the second is MPT. “Calcium cycling” refers to the depletion of mitochondrial energy stores, by the futile shuttling of calcium in and out of the mitochondrial matrix by the mitochondrial calcium transporters. In some models of calcium and/or oxidant mediated injury, calcium cycling appears to explain most or all the mitochondrial injury observed. In other models, calcium cycling appears to play minor roles, and the dominant mechanism seems to be the induction of a proteinaceous pore (32). Furthermore, in some models of mitochondrial injury, calcium cycling contributes to (or perhaps is the primary cause of) induction of a MPT. It is, therefore, not surprising that in many cases both cycling-dependent and cycling-independent processes are likely involved in MPT induction.

Opening MPT in the high conductance mode appears to be irreversible and is linked to cell death. It results in osmotic changes and mitochondrial swelling leading to straightening of the mitochondrial cristae. This in turn leads to a rupture of the outer mitochondrial membrane since the inner membrane is larger than the outer membrane. Rupture of the outer membrane releases apoptosis including factor (AIF) and cytochrome c into the cytoplasm (33). These factors then initiate apoptotic cell death by activation of a cascade of caspases. Activation of MPT also plays an important role in excitotoxic cell death in vitro since NMDA-induced mitochondrial depolarization and cell death are antagonized by cyclosporin A. A role in vivo is suggested by the finding that cyclosporin A can inhibit ischemia-induced cell death in the hippocampus (34). Furthermore, bcl-2 inhibits opening of the MPT and release of cytochrome c from mitochondria (35). Bcl-2 resides in the outer mitochondrial membrane and at contact points between the inner and outer membranes.

Stabilization of mitochondrial creatine kinase by creatine inhibits activation of MPT. Proposed components of MPT are hexokinase 1, a voltage-dependent anion channel (porin), the peripheral benzodiazepine receptor and bcl-2 in the outer membrane, mitochondrial creatine kinase in the intermembrane space and the adenine nucleotide transporter in the inner membrane. Drugs that bind to the translocator, such as atractyloside and bongkrekic acid, are respectively activators and inhibitors of MPT. Complexes of hexokinase, mitochondrial creatine kinase, porin and the adenine translocator can be isolated from rat brain (36,37). When the isolated complexes were reconstituted in phospholipid vesicles, calcium loading re-
leased preloaded malate, which was in turn inhibited by cyclosporin, suggesting that the complex constitutes the MPT pore. The calcium-dependent opening of MPT was inhibited by ADP and ATP (36). When creatine kinase was present in its octamer form calcium did not cause activation of MPT, whereas dissociation of creatine kinase resulted in calcium-induced activation of MPT. The inhibitory effect of cyclosporin A on MPT appeared to be a direct interaction with the creatine kinase dimmer, stabilizing it in the octamer form (36). Only the octamer form of mitochondrial creatine kinase is able to interact with the two boundary membranes.

The addition of creatine or cyclocreatine to mitochondria inhibited increases in respiration induced by atractyloside which, in the presence of calcium, activates MPT (38). This was thought to be due to stabilization of the mitochondrial creatine kinase in an octameric form which inhibits the MPT. One potential mechanism by which creatine may exert neuroprotective effects in vivo is, therefore, by stabilizing MPT.

MITOCHONDRIAL ABNORMALITIES IN ALS PATIENTS

There is substantial other evidence implicating mitochondrial dysfunction in sporadic ALS. Mitochondrial abnormalities have been found in liver biopsies from individuals with sporadic ALS (39, 40). Muscle biopsies of individuals with sporadic ALS also show increased mitochondrial volume and calcium levels within the mitochondria (41). Peripheral blood lymphocytes from individuals with sporadic ALS show increased cytosolic calcium and impaired responses to inhibitors of oxidative phosphorylation (42). A recent study showed that there was reduced cytochrome oxidase activity in anterior horn motor neurons of patients with sporadic ALS, while succinate dehydrogenase activity, which is encoded by the nuclear genome, showed normal activity (43).

There is also evidence for mitochondrial DNA abnormalities, which may contribute to observed changes in electron transport activities. An out-of-frame mutation of mitochondrial DNA-encoded subunit I of cytochrome c oxidase was reported in an individual with otherwise typical motor neuron disease (44). An interesting technique for attempting to determine whether mitochondrial DNA plays a role in producing electron transport activity defects is to utilize cybrid cell lines. These are produced by fusing a patient’s platelets into cell lines that are depleted of mitochondria. This then results in the mitochondria being present in a different nuclear context. If an electron transport defect is found, it implies that it is encoded on the mitochondrial genome. A study of ALS cybrids showed a significant decrease in complex I activity as well as trends toward reduced complex III and IV activities and an increase in free radical scavenging enzyme activities (45).

REFERENCES

4. Borchelt DR, Lee MK, Slunt HS et al. Superoxide dismutase 1 with mutations linked to familial amyotrophic lateral scl-
Mitochondria and oxidative damage in ALS


38. O’Gorman E, Beutner G, Dolder M, Ko-


