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News and Commentary

ALS: astrocytes take center stage, but must they share the spotlight?

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Recent research has implicated non-neuronal cells in the degeneration of motor neurons in amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease. In two articles published in *Nature Neuroscience*, Nagai *et al.*[1] and Di Giorgio *et al.*[2] present *in vitro* data that link astrocytes to motor neuron cell death in cellular models of ALS initiated by mutant Cu/Zn^{2+} superoxide dismutase 1 (*SOD1*). Furthermore, the results indicate that mutant SOD1 astrocytes release one or more toxic substances that selectively kill both mutant and wild-type motor neurons. These discoveries signal new directions for ALS research and the search for effective treatments.

Amyotrophic lateral sclerosis is a progressive neurodegenerative disease characterized by the selective degeneration of upper and lower motor neurons. Respiratory failure is the most common fatal event, usually occurring 1–5 years after disease onset.[3] The typical age of onset is midlife, ranging from age 45 to 60, with a lifetime risk of 1 in 1000.[4] The mysterious and relentless disease has long frustrated patients and researchers alike. Although the French clinician Jean-Martin Charcot first identified ALS 135 years ago, to this day scientists have been unable to determine the cause or the underlying mechanisms of the sporadic form of the disease, which is the most prevalent manifestation and of unknown etiology. However, we do know that mutations in *SOD1* cause one rare form of familial ALS, accounting for approximately 2% of the total cases in humans. Furthermore, most of our knowledge of the pathology of ALS derives from studies of mutant SOD1 animal models. The hope is that studies of these rare familial ALS cases will lead to a better understanding of the mechanisms underlying the more common sporadic disease.

In Search of a Mechanism

The known function of SOD1 is to neutralize superoxide radicals. Because superoxide radicals are highly toxic to cells and implicated in neurodegeneration, an attractive hypothesis was that a loss of function in human SOD1 mutants, resulting in decreased enzyme activity and increased oxidative stress, caused neurodegeneration. However, numerous observations prompted the notion that the mutations cause motor neuron death by a toxic gain of function.[4,5] For example, SOD1 null mice do not develop disease.[5] In addition, a simple increase in wild-type SOD1 expression does not cause disease.[6] Thus, ALS-like symptoms develop in mice irrespective of the level of SOD1 free radical-scavenger activity.[6–9]

While the search for the toxic mechanism of SOD1 mutation originally focused on motor neurons, more recent studies began to implicate non-neuronal cells and non-cell-autonomous pathways. These findings were the first to hint at the possibility of a role for glia in the disease mechanism. This new focus culminated in a study by Clement *et al.*[10], who generated chimeric mice composed of mixtures of normal and mutant SOD1 cells to clarify the role of non-neuronal cells in motor neuron disease. Interestingly, motor neuron loss in the chimeras was asymmetric, even when all of the motor neurons were mutant. The side with higher neuronal survival had a higher proportion of wild-type non-neuronal glia. In addition, some wild-type neurons accumulated ubiquitinated inclusion bodies, which are characteristic of motor neuron disease. These results clearly indicated that non-neuronal cells such as glia contributed to motor neuron degeneration. But which type of glia, microglia or astrocytes, cause motor neuron death?

An elegant *in vivo* study by Boillee *et al.*[11] showed that mutant SOD1 microglia had no effect on disease onset, but played an important role in disease progression. They showed that limiting the damage of mutant SOD1 to microglia slowed, but did not prevent, the disease process. These findings also suggest that different cell types might mediate different phases of the disease, such as initiation and progression. Initiation likely requires direct damage of motor neurons (cell-autonomous), but disease progression depends on non-neuronal cell types such as microglia.

Another study by Beers *et al.*[12] demonstrated that transplantation of mutant SOD1 microglia into mice that are unable to develop precursors of microglia did not cause motor neuron disease. However, in mutant SOD1 mice, wild-type microglia had a protective effect, slowing down ALS-like disease progression. Furthermore, the same study shows that activated mutant SOD1 microglia release more neurotoxins, such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), than do wild-type microglia.

Besides this documented role of microglia in the progression of ALS-like disease, the Clement study, together with findings by Bruijn *et al.*, led to speculation that astrocytes are also potential culprits in familial ALS pathogenesis. The Bruijn

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et al.[8] study found that SOD1 mutant mice develop inclusions, rich in SOD1 polypeptides, in astrocytes. These astrocytic inclusions appear well before similar inclusions in neurons, pointing to the possibility of astrocyte dysfunction. Thus, mounting evidence suggested that there was a strong non-cell-autonomous component to SOD1-linked ALS motor neuron degeneration and astrocytes might well be another murderous component. However, whether astrocytes are offending cells and the method of their action remain unresolved. The two companion papers published in *Nature Neuroscience* provided one missing piece to the puzzle by definitively linking astrocytic toxicity to motor neuron death *in vitro*.[1,2] But, while the papers provided many answers, they also raised many questions for further investigation.

On the Trail of a Killer

Nagai *et al.*[1] utilized a novel coculture system comprised of an astrocyte monolayer and primary spinal cord or embryonic stem cell-derived motor neurons to study the interaction between astrocytes and neurons in neurodegeneration. The research team, led by Przedborski, produced a series of experiments that clearly link mutant SOD1 expression in astrocytes to selective motor neuron death. The investigators observed that non-transgenic motor neurons grown in the presence of mutant SOD1 astrocytes showed the same morphological alterations as mutant SOD1 motor neurons grown in the presence of non-transgenic astrocytes. In addition, loss of motor neurons was greatest in the presence of mutant SOD1 astrocytes. Interestingly, the effect of wild-type SOD1 astrocytes on the survival of motor neurons did not differ from that of non-transgenic astrocytes, eliminating the possibility that simple overexpression of SOD1 caused toxicity. Finally, a previous study reported that mutant SOD1 microglia, unlike the mutant astrocytes in the Nagai study, did not cause death of wild-type motor neurons in a mouse model.[12] Taken together, these results indicate that astrocyte expression of mutant SOD1 may represent the much anticipated non-cell-autonomous mechanism of motor neuron degeneration in familial ALS.

Having identified astrocytes as potential mediators of the non-cell-autonomous mechanism of ALS motor neuron degeneration, Nagai *et al.* turned their attention to the mechanism of astrocyte toxicity. They found that the number of surviving motor neurons cultured with media preconditioned by mutant SOD1 astrocytes was significantly lower than the number of surviving motor neurons cultured with media preconditioned by wild-type astrocytes. These findings support the hypothesis that mutant astrocytes release a soluble toxic agent or agents that are specifically toxic to motor neurons, as the mutant astrocyte-preconditioned media inhibited motor neuron survival but did not alter the viability of interneurons, GABAergic neurons, or dorsal root ganglion neurons. The reason for the selective vulnerability of motor neurons might be the activation of specific cell death pathways involving Fas and NO.[13]

The results of the companion study largely confirm the findings of Nagai *et al.*[1] Using embryonic stem cell lines, Di Giorgio *et al.* linked mutant astrocyte activity to the selective death of motor neurons. The most significant difference in results between the two studies was the relative sensitivity of mutant motor neurons and wild-type motor neurons to astrocyte toxicity. The Nagai study found that after 7 days, the expression of mutant *SOD1* in motor neurons did not increase neuronal cell death or morphometric changes characteristic of degeneration when compared to expression in astrocytes alone.[1] On the other hand, the Di Giorgio[2] study reported an increase in mutant motor neuron death 2–4 weeks after plating. However, these findings are not contradictory, as the Nagai study did not measure survival rates beyond 2 weeks. Furthermore, while non-cell-autonomous pathways play a role, these results also indicate that there is a cell-autonomous component of motor neuron death and that the mutant SOD1 also has some overt effects on motor neurons.

These new findings implicate astrocytes as the murderous cells in familial ALS. In addition, we now suspect that the astrocytes release a soluble toxin that selectively damages motor neurons. While these discoveries are an important step toward solving the riddle of ALS, many questions remain. What are the toxic factors released by mutant astrocytes? Which signal transduction pathways are induced? What are the limitations of these new studies? What is the ultimate impact of these findings for ALS and other neurodegenerative disorders?

Astrocytes: Poisonous Neighbors

Clarifying the toxic nature of mutant SOD1 will be an important step toward uncovering the mechanism of motor neuron death. An early hallmark of disease progression in human ALS and mouse models is the presence of aggregates containing SOD1 in astrocytes followed by aggregate formation in neurons.[8] Inclusions are 10 times more abundant in astrocytes than in neurons, supporting the hypothesis that astrocytes are the major target of mutant SOD1. The origin and effect of these aggregates is still up for debate. Whether aggregates are toxic is unclear. One view is that some mutations lead to slight unfolding of the protein permitting increased entry of peroxynitrite to the SOD1-bound copper, resulting in tyrosine nitration.[14] The second view holds that the mutants catalyze formation of hydroxyl radicals via the Fenton reaction. Hydroxyl radicals in turn may damage cellular targets including SOD1 itself, mitochondria, and glutamate transporters. Oxidation of SOD1 and other targets results in release of bound copper and zinc, which can increase toxicity by inhibiting mitochondrial function and energy supply. Besides aberrant chemistry, misfolded mutant SOD1 protein may inhibit the proteasome, mitochondria, and the endoplasmic reticulum.[15,16] Finally, abnormal protein aggregation is another possible mechanism of action of mutant SOD1 protein.[17] In summary, mutant SOD1 may modify astrocyte function by altering the free-radical milieu, ion homeostasis, protein degradation, energy supply, and signal transduction pathways.

Although Nagai and colleagues demonstrated that mutant SOD1 astrocytes release a toxic substance(s), the identity of the soluble factor is still in question. Making strides toward addressing this problem, Nagai *et al.* excluded the involvement of Fas ligand and caspases in stem cell-derived motor neuron

death. Prior research casts particular suspicion on three substances: glutamate, NO, and nerve growth factor (NGF).

Astrocytes have an intricate relationship with neurons and act as sponges to mop up the sea of glutamate, an excitatory neurotransmitter, following synaptic transmission. A family of membrane-bound glutamate transporters, excitatory amino-acid transporter/glutamate transporter (EAAT2/GLT1) and glial high-affinity glutamate transporter, perform this function. Mutant SOD1 in astrocytes may inactivate glutamate transporters[18] causing motor neuron death by increased firing rates, excessive Ca^{2+} entry, activation of nitric oxide synthase (NOS), and NO and $ONOO^-$ formation. Supporting this view is the finding that familial ALS patients and mutant SOD1 mice exhibit elevated glutamate levels and decreased levels of an astrocytic glutamate transporter EAAT2.[19,20] In addition, the antibiotic β-lactamin increases EAAT2 expression, delays motor neuron death, and increases survival of SOD1 mutant mice.[21,22] These observations led to the speculation that toxicity by excitatory glutamate might be at least one of the mechanisms of mutant SOD1 astrocyte-mediated damage of motor neurons. Also, excitotoxicity appears to be a common link between sporadic and familial ALS.[3] Surprisingly, Nagai *et al.*[1] did not detect any increase in extracellular glutamate levels or impairment of glutamate uptake in mutant SOD1 astrocyte cultures. Furthermore, inhibition of AMPA/kainite receptors by 6-cyano-7-nitro-quinoxaline-2,3-dione did not alleviate mutant SOD1 astrocyte-induced motor neuron death. However, because these observations were made entirely *in vitro*, the possibility remains that the coculture system may not accurately recapitulate the situation in ALS patients or mutant SOD1 mice. Compensatory signaling pathways might get upregulated during culturing. In addition, these findings do not exclude the involvement of glutamate receptors of the *N*-methyl-D-aspartate subtype. Thus, whether glutamate is one of the factors released from dysfunctional astrocytes in ALS remains an open question.

Alternatively, mutant SOD1 astrocytes might release excessive NO. Nitric oxide is a gas and neurotransmitter that is beneficial under normal conditions. However, when produced in excess, NO combines with superoxide anions (O_2^-) to form $ONOO^-$, which is a highly neurotoxic radical. Astrocytes of mutant SOD1 mice and ALS patients exhibit increased levels of NOS,[13] inhibition of NOS prevents motor neuron loss,[23] and at least one NOS inhibitor prolongs the life span of SOD1 mutant mice.[24] In addition, astrocyte production of NO or $ONOO^-$ induces mitochondrial injury[25,26] and motor neuron apoptosis requires astrocyte production of NO.[27] ALS patients, mutant SOD1 mice, and motor neurons undergoing apoptosis, all exhibit increased levels of nitrotyrosine, the nitration of tyrosine residues by $ONOO^-$ altering cellular proteins.[23] Taken together, these findings create the possibility that NO is one of the factors that diffuses from mutant SOD1 astrocytes to trigger neighboring motor neuron cell death. Selective destruction of motor neurons triggered by NO is plausible and would coincide with the results of the Nagai *et al.* study. However, the potential role of NO in astrocyte toxicity requires further investigation.

Another potential toxic agent is NGF. A previous study indicated that astrocytic production of NGF when NO was present caused motor neuron death.[28] This may be applicable to the situation of motor neuron injury in ALS. While Nagai *et al.*[1] found that neutralizing antibodies against NGF were not protective, culture conditions including the presence of glial-derived neurotrophic factor, could have prevented NGF toxicity in the experimental system by activating survival pathways.[28] Thus, we cannot exclude NGF as a potential culprit in astrocyte toxicity.

A Need for Caution?

While the elucidation of the non-cell-autonomous component of motor neuron death in familial ALS is certainly an important discovery, there is reason for caution in evaluating its ultimate impact on the search for new treatment options for ALS. First, the Nagai and Di Giorgio findings were completely *in vitro* and *in vivo* studies were far less definitive. For example, transgenic mice expressing mutant SOD1 only in astrocytes did not develop motor neuron degeneration[29] and mutant SOD1 microglia contribute to motor neuron death,[12] calling into question the ability of astrocytes alone to initiate motor neuron degeneration *in vivo*. Keeping this in mind, a potential concern is that the mutant SOD1 astrocyte preparations used in the Nagai study may have contained a small percentage of contaminating activated microglia, which may release NO. In addition, to compare the relative toxicity of mutant astrocytes and microglia, it would be interesting to investigate if mutant microglia are toxic to wild-type motor neurons in the coculture system. Second, an *in vivo* study by Gould *et al.*[30] has shown that deletion of Bax in a mouse model of ALS significantly increases motor neuron survival, but does not significantly increase mouse survival. In other words, progression of ALS and eventual death appeared to result from damage to distal motor axons, not from activation of the cell death pathway. The Gould study is particularly relevant because experimental results indicate that astrocyte toxicity is Bax-dependent.[1] Thus, if astrocyte toxicity is Bax-dependent and its main manifestation is motor neuron death, is this non-cell-autonomous mechanism a primary cause of ALS symptoms or a secondary response? Of course, astrocytes may act in other ways and may promote the primary denervation of neurons through damage to distal motor axons, but this is yet to be proven and remains a question for further investigation. Finally, while several studies suggest a toxic role for astrocytes in sporadic ALS,[31–33] the specific role is not clear and thus we are yet to determine how relevant this new research is to the sporadic disease. In addition to the contribution of astrocytes to the disease mechanism, there seem to be specific contributions from microglia and motor neurons that we need to examine more closely.

Looking Ahead

New discoveries in ALS research, such as the ones discussed here, illustrate the complexity of the disease. We now know that there are active cell-autonomous and non-cell-autonomous mechanisms that cause motor neuron damage and death. Accordingly, successful treatment will likely require attention to multiple pathways and cell types. Though the new studies indicate a significant contribution from astrocytes, it is unlikely that sole targeting of astrocytes *in vivo* will suffice. Thus the therapeutic use of stem cells will be difficult, because the concerted action of several cell types appears to bring

about the disease, raising the question of which cell type the stem cells should replace. In addition, the mechanism of astrocyte toxicity is far from clear and, while NO and NGF appear to be a strong candidates for involvement, we have not confirmed their participation, nor have we excluded several other potential toxins. Identifying the toxic agent(s) released by astrocytes and microglia would, however, provide a much needed diagnostic tool. In essence, the new findings give us a new avenue for exploration and a renewed understanding that we must continue to focus on the intricate network instead of isolated components in our search for an ALS cure. Finally, lessons learned here may prove to be applicable to other neurodegenerative diseases.

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