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Control of neuroinflammation as a therapeutic strategy for amyotrophic lateral sclerosis and other neurodegenerative disorders

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Abstract

Neurodegenerative diseases, Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis (ALS) are progressive and devastating disorders of the nervous system without cure. Although a number of distinct, but not mutually exclusive, mechanisms can affect disease pathogenesis, neuroinflammation stands in common. It commonly occurs as a consequence of oxidative and excitotoxic neuronal damage, mitochondrial dysfunction and protein aggregation. Thus, it is believed drugs that modulate inflammation may combat disease progression. Such strategies include those commented on in this report by Arie Neymotin, et al. demonstrating lenalidomide's anti-inflammatory and neuroprotective responses in the G93A mutant superoxide dismutase-1 mouse model of ALS (Neymotin, et al., 2009). While anti-inflammatory interventions may be required, they may not be sufficient, to positively affect clinical outcomes. The targeting of combinations of pathogenic events including clearance of disaggregated proteins together with neuroprotective and immune modulatory strategies may all be required to facilitate positive disease outcomes. This may include the targeting of both innate and adaptive neurotoxic immune responses. The commentary is designed to summarize the promises and perils in achieving immunoregulation for brain homeostatic responses and inevitable therapeutic gain. Promising new ways to optimize immunization schemes and measure their clinical efficacy are discussed with a particular focus on ALS.

Keywords

microglia; pro-inflammatory cytokines; T cells; neuroprotection; anti-inflammatory medicines; neurodegenerative disorders

Introduction

Control of microglial inflammatory activities has recently come full circle in studies of neurological disease. First recognized as a response to injury microglia are now believed to be a driver for therapeutic benefit (Colton, 2009). Microglia serve to clear debris and affect immune repair responses. Following activation, they release a broad range of pro-inflammatory factors that include tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-12,

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chemokines, proteases, glutamate, various free radicals, and redox proteins (Shie and Woltjer, 2007, Suzuki, et al., 2005). Autocrine and paracrine amplifications of these microglial secretions define an activated state (Adams and Hamilton, 1987), support leukocyte cell entry into the central nervous system (CNS) and across the blood brain barrier (BBB), and modulate neurotoxic factor production. The latter include proinflammatory factors including cytokines that lead to the generation of chemokine gradients. These modulate cell adhesion molecules, affect changes in leukocyte shape and volume, and pull activated leukocytes to sites of neuroinflammation (Babcock, et al., 2003). Following this proinflammatory phase, microglia can elicit anti-inflammatory and neurotrophic activities leading to neural repair (Edwards, et al., 2006, Mosser, 2003). A third microglial state is now recognized and denoted as "acquired deactivation" characterized by inhibition of cytokine production and major histocompatibility complex (MHC) class II antigens. This makes microglia less effective at antigen presentation (Gordon, 2003, Williams, et al., 1996). Acquired deactivation follows microglial exposure to IL-10 and transforming growth factor beta (TGF-β), which are released by anti-inflammatory regulatory T cells recruited to the inflammation site during adaptive immune responses (Gordon and Taylor, 2005). Each of these microglial functional states is linked, in one way or another, to disease.

A key component in the pathobiology of neurodegenerative diseases involves oxidative stress and mitochondrial dysfunction; both of which are an effect of microglial inflammation (Hensley, et al., 2006). Each can substantively affect neuronal function directly or through speeding the accumulation of misfolded proteins that are not effectively cleared and by activating microglia (Benner, et al., 2008, Stone, et al., 2009). Consequently, accumulated protein aggregates trigger microglial activation and neuroimmune responses, which in turn, induce inflammation and oxidative stress leading to neurodegeneration. Alternatively, neuroinflammation may be a direct response to protein aggregation (Frank-Cannon, et al., 2009). Adaptive immune responses, initiated or driven by activated microglia, become chronic, leading to the further exacerbation of neuronal function. Microglia can affect neurotoxic activities through their expression of the fractalkine receptor (CX3CR1). CX3CR1 deficiency was shown to dysregulate innate immune responses, resulting in neurotoxicity. In PD and ALS models *Cx3cr1*$^{-/-}$ mice showed more extensive neuronal cell loss than *Cx3cr1*$^{+}$ littermate controls (Cardona, et al., 2006). These results, taken together, have given rise to the notion that inhibition of inflammatory activities may improve clinical outcomes for a broad range of neurodegenerative disorders including Alzheimer's and Parkinson's disease (AD and PD) and amyotrophic lateral sclerosis (ALS) (Appel, 2009, Neymotin, et al., 2009, Stone, et al., 2009, Walker and Lue, 2007). Nonetheless, clinical interventions with anti-inflammatory medicines for neurodegenerative disorders have met with mixed outcomes in disease progression (Banerjee, et al., 2008, Bhatt and Gordon, 2007, Chen, et al., 2005, Cudkowicz, et al., 2006, Etminan, et al., 2003, Henkel, et al., 2009, Popat, et al., 2007). This could be due to many divergent reasons including that inflammation, which is more chronic in nature, may have both trophic and toxic outcomes; that responses later in the course of disease may have little to do with the pathogenic process; that other mechanisms for neural impairments dominate; and/or that any immune modulatory agent cannot work in a vacuum without neuroprotective and repair processes being operative simultaneously. This was demonstrated recently by Harraz and colleagues (Harraz, et al., 2008). The investigators showed that mutant superoxide dismutase-1 (SOD1) mutants, in human cell lines, stimulate NADPH oxidase (Nox) by binding to Rac1 resulting in neurotoxic ROS overproduction. The authors concluded that reduction in microglia ROS with apocynin or by elimination of Nox extends survival in ALS mice. Protein aggregation into plaques may also prove beneficial by sequestering potentially toxic oligomers as shown in models of neurodegenerative disease (Kayed, et al., 2007, Selkoe, 2007).

Complete deletion of the SOD1 gene does not cause motor neuron disease in mice, while mutant genes do, when expressed no matter if the enzyme is functional. Thus, the mutated protein

exhibits some toxic function. Experiments to date have not identified that function. However, a final common pathway leading to cell death is activation of a caspase. This triggers a cell-based cascade leading to apoptotic cell death of these neurons. Supporting this idea is that caspase inhibitors have been shown to slow disease in animal models (Pasinelli, et al., 2000, Raoul, et al., 2002). Thus, regardless of the primary events, aggregated and oxidized proteins trigger neuroinflammatory processes that are initiated through microglial immune responses.

Misfolded protein aggregates and dysregulation of innate immunity

What is in fact responsible for microglial activation in ALS and other neurodegenerative diseases? The answer lies, in part, in protein aggregates and pre-aggregated protofibrils. Indeed, hallmarks of neurodegenerative diseases are misfolded proteins. Under homeostatic conditions misfolded or damaged proteins are cleared by the ubiquitin-proteasome pathway. However, in AD, PD and ALS normally soluble amyloid beta (Aβ), α-synuclein (α-syn), and SOD1 become misfolded and form insoluble plaques and intracellular inclusions (Olanow, et al., 2009). Although the etiology of the aggregates remains controversial, modification by reactive oxygen species (ROS), reactive nitrogen species and reactive carbonyl species can alter their protein conformation (Stone, et al., 2009). Protein vulnerability to oxidative stress in the brain commonly results from high oxygen demand and relatively low anti-oxidant defense mechanisms. Moreover, such protein aggregates, including protofibrils are commonly released into the extracellular environment from damaged or dying neurons and as such, exert pathogenic effects (Kakimura, et al., 2001, Lee, et al., 2005, Sung, et al., 2005).

Recent studies employing genomic, proteomic, and physiological analyses demonstrate that aggregated proteins such as nitrated-α-syn (N-α-syn) shifts innate immune responses from neurotrophic to neurotoxic. Misfolded protein stimulation of microglia induces a neurotoxic phenotype marked by increased transcription of genes and whose expression of inflammatory and redox protein products are correspondingly upregulated (Reynolds, et al., 2008, Reynolds, et al., 2008). In particular, how mutant SOD1 affects microglial activation and subsequently injures neurons may revolve around extracellular ATP acting through purinergic P2 receptors. This represents a well-recognized neuron-to-microglia alarm signal. Up-regulation of P2X(4), P2X(7), and P2Y(6) receptors, and down-regulation of ATP-hydrolyzing activities are found in mutant SOD1 microglia. This potentiation of the purinergic machinery reflected an enhanced sensitivity mainly to 2′-3′-O-(benzoyl-benzoyl) ATP, a preferential P2X(7) receptor agonist, and translated into morphological changes, enhanced TNF-α and cyclooxygenase-2 expression, and finally toxic effects exerted on neuronal cell lines by microglia expressing mutant SOD1 (D'Ambrosi, et al., 2009).

Searching for ways to attenuate disease

Nonetheless, neurodegenerative diseases are all rapidly progressive with few therapeutic options. This has led to the search for better compounds with broad activities against ongoing pathogenic events. ALS is a progressive neurodegenerative disease characterized by the selective loss of lower and upper motor neurons. The pathology is imputable in approximately 2% of cases to mutations in the ubiquitous enzyme Cu, Zn SOD1. Thus, for ALS, the experimental systems used for testing were in mice overexpressing human SOD1 with the G93A mutation. This included not only anti-inflammatory agents, but also anti-epileptics, antibiotics, anti-oxidants, protease inhibitors, growth factors, and the elimination of disaggregated proteins, reviewed in (Benatar, 2007, Vincent, et al., 2008). A few examples, though not a complete list of such animal investigations are given to provide a prospective on the topic. Rasagiline, an anti-apoptotic compound with neuroprotective potential, can improve motor function and increase animal survival. Such responses were enhanced with riluzole (Kupershmidt, et al., 2009, Waibel, et al., 2004). Ceftriaxone, a β-lactam antibiotic, can

improve survival and behavior symptoms in SOD1-G93A mice mediated by increased glutamate transporter expression (Rothstein, et al., 2005). The peroxisome proliferator-activated receptor gamma (PPARγ) agonist, pioglitazone, a thiazolidinedione anti-diabetic agent, with reported anti-inflammatory properties, improved muscle strength, body weight and survival as well as demonstrated delayed disease onset when compared to nontreated SOD1-G93A mice (Schutz, et al., 2005). Copaxone, an immune modulatory drug has also been shown to affect survival; however, later results have not been confirmed (Gordon, et al., 2006, Haenggeli, et al., 2007, Schwartz and Ziv, 2008).

The manganese porphyrin was shown to extend survival with greatest improvement seen when the drug is given at symptom onset (Wu, et al., 2003). The current study and one previously published (Kiaei, et al., 2006) demonstrate that thalidomide and lenalidomide cannot directly extend survival, but the effects are through notable reductions in TNF-α and fibroblast-associated cell-surface ligand (FasL) along with other cytokines known to affect neuronal function and viability (Benatar, 2007, Kiaei, et al., 2006, Neymotin, et al., 2009). Work from the same group has also demonstrated additive neuroprotective effects of histone deacetylase inhibitor and a catalytic anti-oxidant in ALS models (Petri, et al., 2006). Indeed, to date, scores of publications have described therapeutic agents that extend the lifespan of this mouse; however, corresponding clinical efficacy remains limited.

Computer modeling analyses of G93A mice were conducted in the quest to better interpret efficacy (Scott, et al., 2008). Compounds reported in animal studies (minocycline, creatine, celecoxib, sodium phenylbutyrate, ceftriaxone, WHI-P131, thalidomide and riluzole) were re-assessed in study designs for optimal power and efficiency as would be predicted in clinical trials. The authors found no survival benefit when agents were administered by their reported routes and doses and concluded that the majority of published effects were noise measures for the distribution of survival rather than actual drug effect. Among studies in which treatment was initiated at the time of symptom onset, subgroup analyses suggested that drugs such as minocycline and Cox-2 inhibitors with an anti-inflammatory mechanism of action, and anti-oxidative agents such as creatine or the manganese porphyrin AEOL-10150, would be most promising for preventative and therapeutic trials respectively in patients with familial ALS. However, this has not yet led to clinical benefit (Bhatt and Gordon, 2007). Indeed, research over the last decade produced a single FDA-approved drug for ALS, riluzole (Festoff, et al., 2003), which have led some to conclude that a single agent may not provide adequate therapeutic efficacy. Trials using combinatorial modalities, including celecoxib-creatine and minocycline-creatine combinations are being considered (Banerjee, et al., 2008). During the past 12 years, 11 double blind placebo-controlled clinical trials, based principally on preclinical success in the SOD1 model, have uniformly failed for the treatment of ALS (www.alsa.org). Thus therapeutic success in the SOD1 mouse model of ALS has not translated into effective therapy for human disease, calling into question the utility of such preclinical data for identifying therapeutic agents that are worthy of further study in humans. This may be due, in part, that ALS may not one but several diseases (Rothstein, 2009).

Harnessing neuroinflammation for therapeutic gain

Defining neuroprotective immunity

Following any neural injury, activated resident microglia and infiltrating macrophages induce an inflammatory response. It is these activated brain mononuclear phagocytes (MP; microglia and blood-derived macrophages) that serve to clear debris from neuronal damage and initiate an immune response. The activated MP secrete neurotoxic inflammatory factors that serve to initiate neurotoxic activities while attracting new inflammatory cells to sites of disease. While it is well established that inflammatory responses are damaging, debate remains about phenotype changes of different MP cell populations and the dynamics of the innate immune

response. Such reactions are highly evolutionary and serve to point out changing phenotypes of MP and the subsequent roles of anti-inflammatory drugs that serve to contain their responses. Two studies investigated such a contradiction. Gensel et al. reported that microinjection of zymosan into the spinal cord generates a neurotrophic gradient that promotes axonal growth toward the injection site through reactive microglia embedded in the region of inflammation. Increasing proximity to the zymosan found abortive growth, phagocytic uptake of degenerated axonal remnants, and cell death, events likely dependent upon infiltrating macrophages since selective depletion of circulating monocytes reduced zymosan-associated pathology (Gensel, et al., 2009). Such findings substantiate a wealth of data from a broad number of biological systems supporting the neurotoxic phenotype of activated blood-derived brain macrophages that infiltrate the nervous system and cause neural injury. Indeed these macrophage populations have long been considered functionally identical, *i.e.*, proinflammatory and destructive.

Interestingly, it was also recently suggested that MP could have distinct functions after spinal cord injury (Shechter, et al., 2009). By labeling monocyte-derived macrophages, the findings showed that these cells elicited enhanced motor recovery while their elimination resulted in increased lesion size possibly through the anti-inflammatory cytokine IL-10. The more likely scenario is more chronic MP activation in areas of injury would elicit a mixed phenotypic response (Wu, et al., 2009). This would best explain the conflict and the overall importance of neuroinflammation control as a therapeutic target.

Targeting innate and adaptive immunity and neuroprotective responses

An effective therapeutic strategy against neurodegenerative diseases should clear misfolded protein aggregates, while targeting both innate and adaptive immune-mediated neuroinflammation. Of note is the fact that immunization schemes, particularly in AD, have failed to suppress proinflammatory microglia. As a result, immunization led to neuroinflammation despite effective clearance of protein aggregates (Greenberg, et al., 2003, Holtzman, 2008, McGeer, 2008, Smith, et al., 2002). Thus, targeting of classically-activated microglia, a source of neurotoxic proinflammatory cytokines and excitotoxic mediators, appeared appropriate and required. These cells, as noted, affect secondary neurodegeneration. This strategy is supported by a plethora of evidence in animal models wherein attenuation of microglial inflammatory responses is protective against nigrostriatal dopaminergic neurodegeneration (Mosley, et al., 2006, Stone, et al., 2009). In the MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) mouse model, immunization with glatiramer acetate (GA, Copaxone, copolymer-1, COP-1), a random amino acid polymer and immunomodulatory agent prescribed for relapsing-remitting multiple sclerosis, induces GA-specific CD4+ regulatory T cells that attenuate activated microglia and protect against neurodegeneration (Benner, et al., 2004, Laurie, et al., 2007).

More recently, we demonstrated the capacity of relatively few CD4+CD25+Foxp3+ natural regulatory T cells (Tregs) to abate MPTP-induced neuroinflammation and ameliorate almost all dopaminergic neurodegeneration in both the substantia nigra and striatum (Reynolds, et al., 2007). *In vitro* experiments further showed that co-culture of Tregs and microglia before stimulation with N-α-syn suppresses microglial responses, as measured by diminished NF-κB activation, proinflammatory cytokine production and oxidative stress (Reynolds, et al., 2007, Reynolds, et al., 2009, Reynolds, et al., 2009). Interestingly, Tregs co-cultured with microglia after stimulation induce upregulation of FasL by Tregs and Fas by microglia, which result in Fas-FasL interactions that lead to caspase 3 activation and increased apoptosis of microglia. Such findings, coupled with known roles of Tregs in maintaining self-tolerance and suppressing myeloid APC function and Teff responses (Ozdemir, et al., 2009), have inspired immunization strategies aimed at increasing Treg number and/or activity.

In addition to being neuroprotective, modulation during immunization may also be reparative and regenerative, and thus afford additional benefits. We have demonstrated that adoptive transfer of COP-1 immune cells increases local expression of glial cell line-derived neurotrophic factor (GDNF) in MPTP-intoxicated mice (Benner, et al., 2004). We observed this again after adoptive transfer of activated Tregs, but not Teff (Reynolds, et al., 2007), which suggests that Tregs may be involved in regulating reparative processes in microglia and astrocytes that contribute to greater than 90% protection of dopaminergic neurons. Indeed, in postmortem PD brains, decreases in neurotrophic factors of neurons have been observed and are suggested to play an important role in the pathogenesis of PD (Imamura, et al., 2005). *In vitro*, GDNF is secreted by activated astrocytes and acts as the most potent factor supporting dopaminergic neurons (Burke, et al., 1998). *In vivo*, GDNF induces dopaminergic nerve fiber sprouting upon injury to the rat striatum (Batchelor, et al., 1999), which becomes markedly reduced once GDNF expression is inhibited by antisense oligonucleotides (Batchelor, et al., 2000). Perhaps GNDF can modulate microglia biology to protect against nigrostriatal neurodegeneration. Studies show that GDNF reduces dopaminergic neuronal death and significantly enhances neuronal function in both MPTP-intoxicated mice and monkeys (Eberhardt, et al., 2000, Gash, et al., 1996, Kordower, et al., 2000). Future studies should explore the mechanisms by which Tregs upregulate GDNF in the context of protection against progression of PD and other neurodegenerative disorders.

Testing therapies with broad screens that include relevant animal models

The mutant SOD1 model exhibits many putative pathogenic mechanisms thought associated with ALS including glial cell pathology, proinflammatory cytokine and chemokine production, oxidative damage, intracellular protein aggregation, mitochondrial dysfunction, caspase-mediated death, and axonal transport dysfunction (Rothstein, 2009). Hypotheses that one or several therapeutics would affect convergent pathways to abate all putative mechanisms may have been shortsighted. Thus, given the clinical similarity of both familial and sporadic ALS, the lack of concordance between SOD1-based animal models and human therapeutic trials calls into question the relevance of the model and the broad array of mechanisms by which it exhibits neurodegenerative processes. This may require development of laboratory models with multiple mechanistic targets or more appropriate animal models, which target single mechanisms and when possible, clinical validation.

Perform proof of concept studies in humans and a relevant clinical trial

Therapeutic proof-of-concept requires substantive pre-clinical testing for which ALS therapeutics has followed conventional laboratory and animal preclinical assessments. Considerations include demonstrating the pathobiological deficit seen in laboratory and animal model screens in humans. However, the complexities of ALS disease etiology and progression and the lack of *in vitro* and *in vivo* models that accurately reflect the human disorder, create a major impediment in the translation of these pathobiological deficits into candidate therapeutics.

A second consideration is the pharmacokinetics of the compound or immune modulator as well as its penetration to the CNS. Ostensibly, this may reflect differences in drug metabolism between animal models and humans; thus, optimal doses and routes in humans that permit maximal amount of CNS penetration may be required for the greatest therapeutic efficacy. Unfortunately, this may be substantially different than those used in preclinical models. One of the more challenging and possibly critical issues for clinical trials is picking the correct dose; yet in many ALS trials, only single doses are evaluated without dose-response comparisons. Additionally, compounds with prior FDA approval for a different application may not be satisfactory for ALS treatment because of poor penetration of the blood-brain barrier. It may become necessary to screen similar compounds in order to yield more candidates or to derive

smaller, more lipophilic compounds with better penetrating capacity and better pharmacokinetics, perhaps even nanoformulated compounds with greater resistance to degradation. Such structural analyses also can be used to predict mechanistic actions, particularly if more biomarkers that better correlate with disease progression and mechanisms were available. Therefore, therapeutics could be considered only wherein the goal is to affect a combination of pathobiological events. Thus, based on our current knowledge of ALS pathobiology, the safest and most efficacious therapeutic(s) would attenuate oxidative stress and neuroinflammation and should provide greater levels of neuronal trophic support and regenerative activities, sustain glutamate balance, promote neuronal energy, improve blood flow, and remove protein aggregates. These should all tested and validated in multiple models prior to subsequent dose- and route-finding clinical studies and subsequent trials that include biomarkers used as surrogate measures in preclinical studies.

Conclusions

Evidence abounds that inflammation plays a major role in the pathogenesis of neurodegenerative disorders. Mediators of inflammation, such as the cytokine TNF-α and its superfamily member FasL, are implicated in apoptosis and are targets for drug development. In the excellent report by Neymotin and colleagues, increased TNFα and FasL immunoreactivity are found in lumbar spinal cord sections of ALS patients and G93A transgenic mice. Neuroprotective affects of lenalidomide attenuated weight loss, enhanced motor performance, and diminished motor neuron cell death. Nonetheless, the question at hand is can the therapeutic activities seen be translated for human benefit? A large number of immune modulatory drugs behave in similar manners, but do not evoke human disease combating responses. Rationales are needed with a clear focus in guiding therapeutic development. This can be realized by targeting not one but multiple pathogenic responses then testing those responses in a broader range of relevant animal model systems. To these ends, such immune modulatory therapies can reach the desired therapeutic result.

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