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Forum Review

On the Relation of Oxidative Stress to Neuroinflammation: Lessons Learned from the G93A-SOD1 Mouse Model of Amyotrophic Lateral Sclerosis

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ABSTRACT

The central nervous system (CNS) presents both challenges and opportunities to researchers of redox biochemistry. The CNS is sensitive to oxidative damage during aging or disease; excellent transgenic models of specific neurodegenerative diseases have been created that reproduce oxidative stress components of the corresponding human disorder. Mouse models of familial amyotrophic lateral sclerosis (ALS) based on overexpressed mutant human Cu,Zn-superoxide dismutase (SOD1) are cases in point. These animals experience predictably staged, age-dependent motor neuron degeneration with profound cellular and biochemical damage to nerve fibers and spinal cord tissue. Severe protein and lipid oxidation occurs in these animals, apparently as an indirect consequence of protein aggregation or cytopathic protein–protein interactions, as opposed to aberrant redox catalysis by the mutant enzyme. Recent studies of G93A-SOD1 mice and rats suggest that oxidative damage is part of an unmitigated neuroinflammatory reaction, possibly arising in combination from mitochondrial dysfunction plus pathophysiologic activation of both astrocytes and microglia. Lesions to redox signal-transduction pathways in mutant SOD1^{+} glial cells may stimulate broad-spectrum upregulation of proinflammatory genes, including arachidonic acid–metabolizing enzymes [*e.g.*, cyclooxygenase-II (COX-II) and 5-lipoxygenase (5LOX)]; nitric oxide synthase (NOS) isoforms; cytokines (particularly tumor necrosis factor alpha, TNF-α); chemokines; and immunoglobulin F_c receptors (FcγRs). The integration of these processes creates a paracrine milieu inconsistent with healthy neural function. This review summarizes what has been learned to date from studies of mutant SOD1 transgenic animals and demonstrates that the G93A-SOD1 mouse in particular is a robust laboratory for the study of neuroinflammation and redox biochemistry. *Antioxid. Redox Signal.* 8, 2075–2087.

INTRODUCTION

AMYOTROPHIC LATERAL SCHEROSIS (ALS) is an age-dependent, fatal motor neuron degenerative disease affecting the motor cortex, brainstem, and spinal cord. ALS may be sporadic (SALS) or familial (FALS). The molecular cause of sporadic ALS is unknown, but the disease is inexorable, with median life expectancy after diagnosis of 3 years, although some individuals may live with the disease for much longer (18, 57). The label "ALS" technically applies only to a disease affecting anterior horn cells plus pyramidal tract involvement (18). As such, ALS affects some 60,000–100,000 individuals worldwide at any given time (18, 57). A variety of clinically similar motor neuron diseases that display a technically different pattern of motor neuron degeneration can be collectively termed MND for "motor neuron diseases."

Approximately 10–15% of ALS cases are familial (heritable) in nature. Of this fraction, some 20–30% are caused by

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mutations in the antioxidant enzyme cytosolic Cu,Zn-superoxide dismutase (SOD1) (19, 65). With rare exceptions, SOD1 mutations produce motoneuron disease in a dominantly inherited fashion. More than 90 different mutations in SOD1 have been found in various kindreds afflicted with FALS (15, 18, 57). The clinical features of an individual with FALS are very similar to those of a patient with SALS, although some mutations predict a more rapid disease progression. The most common mutations associated with FALS are the A4V mutation (valine substituted for alanine at position 4 in the protein sequence) and the G93A mutation (alanine substituted for glycine at residue 93) (18, 57). These are associated with lifespan expectations of 2–5 years after diagnosis in the case of G93A, and ~9 months for A4V. Both sporadic and familial ALS typically are first seen at 40–50 years of age (57).

It is not known with any certainty how the SOD1 mutations engender the clinical manifestation of FALS. Data from human studies and rodent genetic models of the disease suggest a close association between oxidative stress and chronic activation of glial cells by exposure to ambient inflammatory molecules. Indeed, murine ALS appears not to be cell autonomous, in that mutant SOD1 produces serious disease only when expressed in both motor neurons and surrounding non-neuronal cells. The chronic, feed-forward cycle of glial cell activation leading to inflammatory cytokine production, microglial proliferation, and neurotoxicity constitutes a phenomenon loosely termed "neuroinflammation."

In recent years, mouse models [and, more recently, a rat transgenic model (41)] of ALS have proven themselves to be convenient and productive laboratories for the study of both redox biochemistry and neuroinflammation. These animals are beginning to shed light on the etiology not only of ALS but also of other neurodegenerative diseases wherein oxidative stress and neuroinflammation are germane. As preclinical tools for the preliminary evaluation of lead pharmaceutical candidates, SOD1-mutant animals are guiding decisions about which drugs to press into human clinical trials. The present review summarizes current knowledge regarding the neuroinflammatory phenotype of murine ALS models, especially the fast-progressing strain of G93A-SOD1 mutant mouse, with special attention given to the pros and cons for using these animals in preclinical drug studies.

SOD1 MUTATIONS CAUSE HERITABLE ALS AND PRODUCE A MURINE MODEL OF MOTOR NEURON DISEASE

The first evidence for Cu,Zn-SOD involvement in ALS came in 1993 with the discovery by Rosen *et al*. (65) that certain cases of familial ALS (FALS) were linked to mutations in the *SOD1* gene. Subsequently, >90 mutations in Cu,Zn-SOD have been discovered in dozens of FALS kindreds (18, 57). With the exception of the D90A mutation (2), these mutations are inherited in an autosomal dominant fashion. Loss of function of SOD would be anticipated to propagate as a recessive trait. Likewise, many of the mutations in *SOD1* do not appreciably diminish the ability of the enzyme to disproportionate superoxide into hydrogen peroxide (discussed further later). Furthermore, mice with a genetic ablation of *SOD1* do not develop blatant neurodegenerative pathology but rather display decreased fertility (40). In contrast, transgenic mice engineered to express the mutant human SOD1 protein have motor neuron disease very similar to human ALS (30). Multiple molecular and histologic similarities have been noted between murine and human ALS, including selective degeneration of motor neurons with marked damage to anterior horn cells and prominent distal axonopathy (22); mitochondrial perturbations including cytochrome *c* release (28); defects in excitatory amino acid transporters (8, 41); and neuroinflammatory correlates (discussed later). Most strains of SOD1-mutant mice develop paralysis within 3–6 months of age, depending on the exact mutant and background of the mouse and the SOD1 copy number. High-copy-number G93A-SOD1 mice that possess ~20 copies of the transgene experience paralysis at ~115 days of age and die within 3 weeks thereafter (29, 79). Mice expressing equivalent levels of wild-type human SOD1, in addition to the normal murine complement of SOD1, are disease free (9, 30, 79).

The most attractive practical feature of SOD1-mutant mouse models is the reproducible, age-dependent nature of disease. Defined molecular, cytologic, physiologic, and behavioral features become manifest in these animals as they age (Figs. 1 and 2). In fast-progressing strains of G93A-SOD1 animals, the entire disease process occurs within 4 months after birth (Figs. 1 and 2). In principle, this age-dependent staging of disease phenomena offers the opportunity to understand the chain reaction(s) that must occur to translate a simple genetic mutation of SOD into full-spectrum neuroinflammatory disease. Additionally, the rapid and reproducible nature of murine ALS allows a relatively expeditious testing of prospective neuroprotective or antineuroinflammatory compounds.

A note should be made regarding gender and background effects of SOD1 mutations. Most G93A-SOD1 mouse studies have used mice of a C57BL/6JxSJL/J hybrid background. When these animals are completely backcrossed to C57BL/6J mice, survival is extended by ~20 days (34). This may be important because a sizable and growing number of drug candidates tested in mixed-strain SOD1-mutant mice extend the life span by 5–20 days, whereas very few pharmacologic or genetic manipulations surpass this apparent 3-week survival limit. Thus, it is possible that many of the positive drug effects seen in C57BL/6JxSJL/J mice actually alleviate susceptibility factors inherent to the Swiss Webster strain contribution. As a further note, female G93A-SOD1 mice live, on average, 3–5 days longer than males only on a mixed background, in which case, female (but not male) longevity can be slightly extended by exercise (34, 74). Therefore, any sensitive study should include both genders in equal numbers and sufficient numbers to parse gender-specific outcomes statistically. Unless otherwise noted, all murine ALS data referred to here that were collected in the authors' laboratories were acquired through studies of the fast-progressing C57BL/6JxSJL/J strain of G93A-SOD1 mouse.

SOD1 mutations produce a gain of toxic function that remains poorly understood

The exact nature of the gain-of-function in mutant SOD1 remains frustratingly evasive. One hypothesis contends that

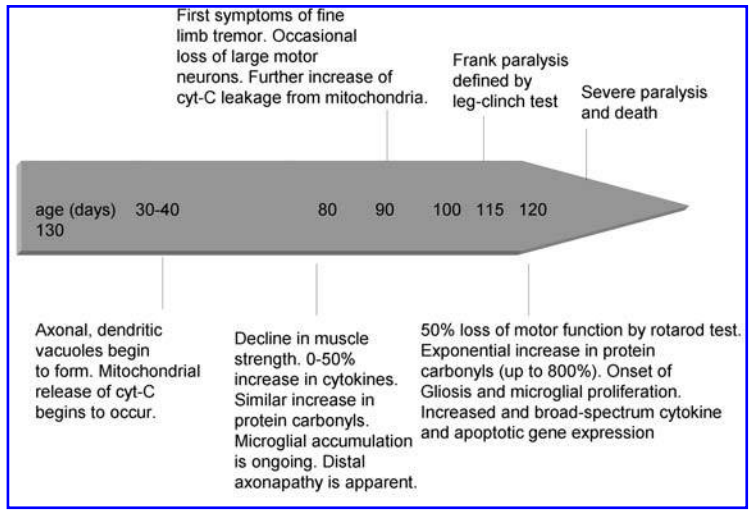

FIG. 1. Time-course for appearance of various major pathological sequelae in the fast-progressing strain of G93A-SOD1 mutant transgenic mouse.

mutant SOD1 may directly promote the generation of toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS). This hypothesis grew out of reports made in 1996, when Valentine and Bredesen (81) noted a gain-of-function of peroxidase activity of the mutant SOD1. This gain-of-function corresponds to a reduced affinity of the mutant enzyme for both Cu and Zn (17, 48), with possible liberation of Cu to perform promiscuous redox reactions. The altered enzyme appeared capable of promoting radical generation in the presence of hydrogen peroxide (H_2O_2), while exhibiting diminished efficiency in $O_2^{\bullet -}$ dismutation (81). Zn-deficient SOD1 tends also to promote nitration reactions (17, 67). The very high (>10 m*M*) K_m for SOD1-associated peroxidase activity, however, argues against the physiologic relevance of this phenomenon (83, 84). Also mitigating against the hypothesis of direct redox toxicity are findings that genetically ablating the copper chaperone protein (64) or mutating Cu binding sites (76) fails to affect disease onset or progression in SOD1-mutant mice. If direct redox activity of SOD1 mutations was responsible for motor neuron disease, one would anticipate that altering the copper content of the protein ought to have some quantitatively significant impact on pathologic parameters.

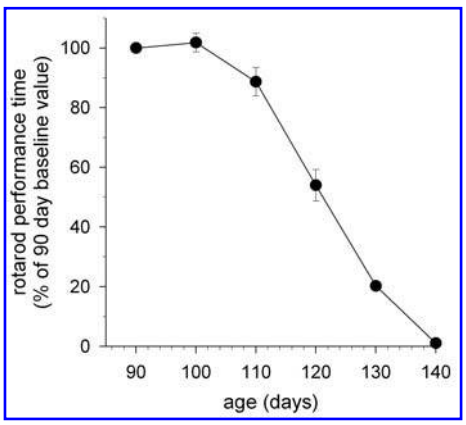

FIG. 2. Age-dependent loss of motor function in G93A-SOD1 mice, as measured by a rotarod performance task (36). Data indicate loss of motor function relative to initial 90-day baseline value of mice that had been trained to the rotarod between 80 and 90 days of age. Each point indicates mean ± SEM for 72 mice (equal numbers of male and female animals were included).

A second prominent hypothesis contends that SOD1 mutants are prone to aggregation inside neurons, thereby facilitating toxicity (3, 9, 76, 78). SOD-positive inclusion bodies are found in ALS neurons, whereas similar, although not SOD1-positive aggregates are present in human sporadic ALS tissue (9, 43, 78). Aggregated SOD1 might induce cell dysfunction and cytotoxicity by multiple mechanisms, such as blockage of the proteosomes, depletion of coprecipitated proteins, or by triggering localized immunoreactions to persistent aggregates. Intriguing new versions of the aggregation hypothesis revolve around findings that SOD1 and especially mutant SOD1 tends to accumulate within mitochondria, in the intermembrane space (39). Aggregation within this vulnerable compartment might explain why mitochondrial vacuole formation (7) and cytochrome *c* release (28, 88) occur relatively early in the presymptomatic phase of disease in SOD1-mutant mice (Fig. 1). SOD1 action in mitochondria might also begin to explain relatively subtle but significant electron transport chain deficits noted in SOD1-mutant animals (49).

Motor neuron death in SOD1-mutant animals is not cell autonomous

The neuron cell autonomy of ALS pathogenesis has been strongly questioned by a number of studies over the past several years. In a study published in 2001, Rouleau's group (62) targeted mutant SOD1 expression to neurons exclusively, rather than ubiquitously, in every cell of the mouse. Mice expressing mutant SOD1 only in neurons fail to develop motor neuron disease. Caroni's group (47) subsequently reported similar findings. In particularly seminal work, Cleveland and colleagues (14) showed that disease progression in mutant SOD1 chimeric mice depends on the extraneuronal expression of mutant SOD1. The survival of chimeric mice was dependent on mutant SOD1 expression in neurons, but also immensely dependent on the presence of mutant SOD1-

expressing nonneuronal cells. The survival of chimeras could be extended by threefold, depending on the ratio of nontransgenic to SOD1 mutant glia, even when all neurons were positive for the mutant SOD1 transgene (14). It should be noted, however, that Elliott's group (21) expressed mutant SOD1 in astroglia specifically without causing motoneuron disease. Thus, it seems likely that mutant SOD1 expression in neurons is necessary but not sufficient to cause murine ALS. Taken together, these studies provide great incentive to consider glial roles in ALS.

Glia, rather than neurons, are the major cell types in the CNS, in terms of number or mass. They fall into three broad categories. Oligodendroglia surround neurons and generate myelin sheaths. Astroglia support, protect, and nourish neurons by secreting growth factors and by consuming excess neurotransmitters such as glutamate that, in high concentration, are neurotoxic. Microglia, the third category of glial cell, are very similar in form and function to the peripheral macrophage. Microglia are the principal immune cells in the CNS (at least, under conditions in which the normal blood–brain barrier is maintained) and play an active role in host defense against invading pathogens and neoplastic cells. Microglia congregate in the vicinity of histopathologic lesions, including senile plaques in Alzheimer's disease (AD) and in horn regions of ALS cord tissue (3, 33). Like macrophages, microglia actively participate in both humoral and cell-mediated defense. Microglia can be activated to generate reactive oxygen and nitrogen species (15); to phagocytose apoptotic cells (12); and to generate proinflammatory cytokines (37). Microglia are competent to bind free immunoglobulin through a high-affinity receptor for immunoglobulin (the FcγRI receptor) and complexed IgG through low-affinity receptors (FcγRII and FcγRIII) (54). They reciprocally communicate with T cells under conditions of acute trauma or during experimentally induced autoimmune demyelination (26, 73), and subpopulations of microglia are highly competent antigen-presenting cells, bearing major histocompatibility complex (MHC) class I and interferon (IFN)γ-inducible MHC class II molecules (11). Although these immune functions are necessary to promote resolution of traumatic injury and to prevent neoplastic transformations, it is highly conceivable that improper microglial responses could promote collateral damage to neurons surrounding sites of chronic microglial activation. For this reason, much research attention is turning toward microglia as major determinants of ALS pathogenesis in humans and mouse models of FALS. The "neuroinflammatory hypothesis" contends that ALS, and perhaps other neurodegenerative conditions, are largely diseases of glia that foster a chronic atypical type of inflammation within the CNS. In this scheme, neurotoxicity is a late-stage consequence of chronic glial activation.

NEUROINFLAMMATION IN HUMAN AND MURINE ALS

Prior to the late 1980s, the CNS was thought to be "immune privileged," and hence concepts of CNS inflammation were largely irrelevant, except for specific situations in which the blood–brain barrier was compromised. Nonetheless, data that have emerged over the past 10–15 years clearly indicate that inflammatory paracrine signaling cascades can begin and propagate within the CNS. Generally these cascades represent a mostly innate immune response confined to the CNS compartment and manifest in the absence of edema and diapedesis. Hence, ambient astrocytes and microglia are the main effector cells with limited to no involvement of peripheral lymphocytes. Detailed reviews of the neuroinflammatory concept have been published elsewhere (50, 52, 58).

Neuroinflammation began to gain credence after seminal work by McGeer and Rodgers (64), who found epidemiologic evidence that long-term treatment with nonsteroidal antiinflammatory drugs reduced the risk for AD, and important findings by Mrak and Griffin (51,52), who documented inflammatory molecules in AD brain. This and subsequent work prompted investigators of other neurodegenerative diseases to question the involvement of immune components in diseases such as ALS that, on the surface, would not appear to be inflammatory in nature. Several studies from the 1990s found peripheral indications of some inflammatory reaction in human ALS patients. For instance, serum and epidermis from ALS patients were found to have greater amounts of interleukin-6 (IL-6) or TNF-α than did control samples (56, 69). Specific cytokines, especially monocyte chemoattractant protein-1 (MCP-1), monocyte colony-stimulating factor (M-CSF), and transforming growth factor β1 (TGF-β1) have been reportedly increased in cerebrospinal fluid from subsets of SALS patients (35, 42). Human tissue studies in ALS are difficult to conduct and interpret because of difficulty in obtaining sufficient sample sizes of spinal cord tissue with adequately short postmortem intervals; low (usually near detection limit) concentrations of cytokines in cerebrospinal fluid; and confounding effects of late-stage lymphocytic infiltrates and secondary reactions to systemic infections during the terminal period of disease.

Inflammatory macromolecules in ALS: immunoglobulins and their receptors

Despite these caveats, some intriguing molecular evidence for CNS-resident inflammatory pathways has been gleaned from human autopsy studies. Appel and colleagues (3) have reported that immunoglobulin (IgG) accumulates in the soma of large motor neurons in human and murine ALS, beginning at presymptomatic phases in the mouse (3), an observation that we have made also in the G93A-SOD1 mouse (unpublished data, K.H.). This IgG likely accumulates through retrograde transport from the neuromuscular junction, because intramuscular injections of IgG are taken up and transported in such a manner (23) and because lymphocytic infiltrates are undetectable in murine ALS spinal cord until the very last stages of disease (3). The origin and antigen specificity, if any, of this motoneuronal IgG remains to be determined, although some evidence suggests reactivity towards L-type voltage-gated Ca^{2+} channels (68), whereas other researchers report enzyme-linked immunosorbent assay (ELISA) data implicating anti-neurofilament IgM in sera from subsets of ALS patients (16). IgG uptake in murine ALS models correlates with increased expression of immunoglobulin Fc receptor subunit expression, especially of the FcR gamma chain (3 and personal observations). Fc receptors for IgG appear lo-

calized mostly to motor neurons (3), although microglia also express these receptors (75).

The biologic significance of Fc receptors on neurons remains to be elucidated, except that one would anticipate the increased expression to produce correspondingly increased cellular uptake of ambient IgG. On macrophages and microglial cells, however, Fc receptors trigger cell activation and ROS in response to antigen-complexed or aggregated immunoglobulins (53, 63, and data presented later). Fc receptors comprise stimulator FcγRI and FcγRIII and a lower-affinity, inhibitor FcRII (63). The RI and RIII receptors share a common γ-chain subunit of 10 kDa that dimerizes to yield a 20-kDa species (63, and see Fig. 9). The γ-chain component is essential for signal transduction through FcγRI and FcγRIII. We have corroborated Appel's observation by measuring increased stimulatory γ-subunit expression at the message and protein level in late-stage G93A-SOD1 mouse spinal cord (Fig. 3). Curiously, in the same lysates, low-affinity FcγRII (the putatively inhibitory subunit, which does not require the γ-chain for function) was decreased at the protein level (Fig. 4). Thus, the G93A-SOD1 mouse CNS may be primed for activation of macrophage/microglial cells via immunoglobulin complexes or other macromolecular complexes, as yet unidentified, that activate the Fc receptor system.

Inflammatory paracrine molecules in ALS: cytokines and eicosanoids

The advent of murine models has facilitated more-controllable studies of neuroinflammation in ALS and more-exacting quantitation of inflammatory cytokine expression, especially at the mRNA level. The first report of cytokine elevation in animal motoneuron disease models was from Ghezzi *et al.* (25), who, in 1998, noted increased TNF-α in the *mnd* mouse. Studies published by two groups in 2001 first reported that message for interleukin (IL-1) and TNF-α were elevated in SOD1-mutant mouse CNS tissue, even at presymptomatic stages of disease (21, 55). Subsequent work by a number of groups including our own extended these findings by measuring disease-associated up- or downregulation of numerous cytokines and chemokines at both the message and protein levels in G93A-SOD1 mouse spinal cord as a function of animal age (13, 36, 37, 85). Very recent studies have found similar cytokine upregulation in the G93A-SOD1 transgenic rat model for ALS (82).

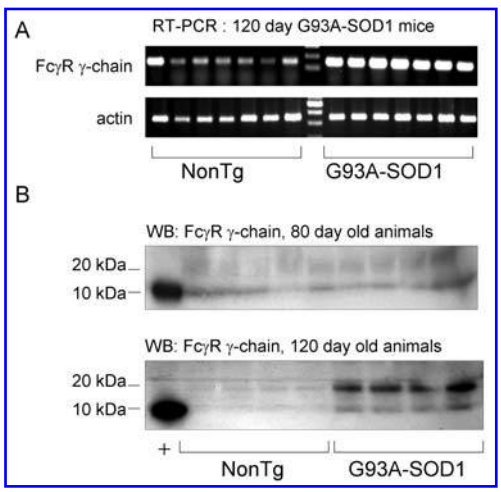

FIG. 3. FcγRI/III γ-chain expression in G93A-SOD1 mouse spinal cord. (**A**) Semiquantitative RT-PCR for γ-chain expression in spinal cord message extracted at 120 days of age [Primers: forward, 5′-CGGGATCCAAGATCCAGGTCCGAAAGGC; reverse, 5′-ATAGTTTAGCGGCCGCCTGGGGTGGTTTTTCATGCTTC; cycling conditions, one cycle of 94°C for 2 min; 25 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min; one final cycle of 72°C for 7 min; product, 143 bp]. Actin was amplified as an internal control. (**B**) Western blots for γ-chain protein in lysates collected at 80 days (upper Western blot) and 120 days of age (lower Western blot). Each lane represents lysate obtained from an individual mouse. RAW 264.7 cell lysate was used as a positive control (+).

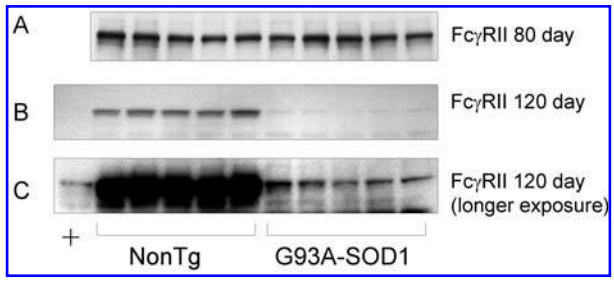

FIG. 4. FcγRII expression in G93A-SOD1 mouse spinal cord at 80 days of age (A) and 120 days of age (B, C). RAW264.7 macrophage cell lysate was used as a positive control (+) in some blots. Each lane represents lysate obtained from an individual mouse.

We have made extensive use of multiprobe ribonuclease protection assays (RPAs) to index inflammation and apoptosis during periods of pathophysiologic stress (36–38, 79, and see Fig. 5). RPAs allow the simultaneous quantitation of multiple mRNA species with 10-fold greater sensitivity than Northern blots. RPAs indicated a macrophage-typical (monokine) pattern of cytokine expression in G93A-SOD1 mouse spinal cords at latter stages of life (36, 37, 79). Several but not all monokines were significantly elevated in the G93A-SOD1 mouse spinal cord at 120 days of age (36, 37). For instance, interleukin 1α (IL-1α) and IL-1β were robustly increased in 120-day-old G93A-SOD1 mice relative to nontransgenic littermates or to mice expressing wild-type human SOD1 (36). We find that TNF-α message is highly overexpressed at 120 days, as is the primary TNF-α receptor TNFRI (37). The aggressive upregulation of the TNF-α system is consistent with recent findings from other laboratories (13, 85). In a microarray study, Yoshihara (85) reported that TNF-α was one of the most highly increased transcripts in G93A-SOD1 mouse spinal cord. Interestingly, we found that transcripts for the IL-1 receptor antagonist (IL1RA) was remarkably increased in G93A-SOD1 mouse spinal cord (36), indicating not only that proinflammatory pathways are upregulated but also that some antiinflammatory components are strongly activated.

RPAs were used to assess the same cytokine mRNAs in spinal cord of 80-day-old animals, before onset of paralysis or frank motoneuron death (36, 37). These data indicate that monokine upregulation precedes and correlates with onset of

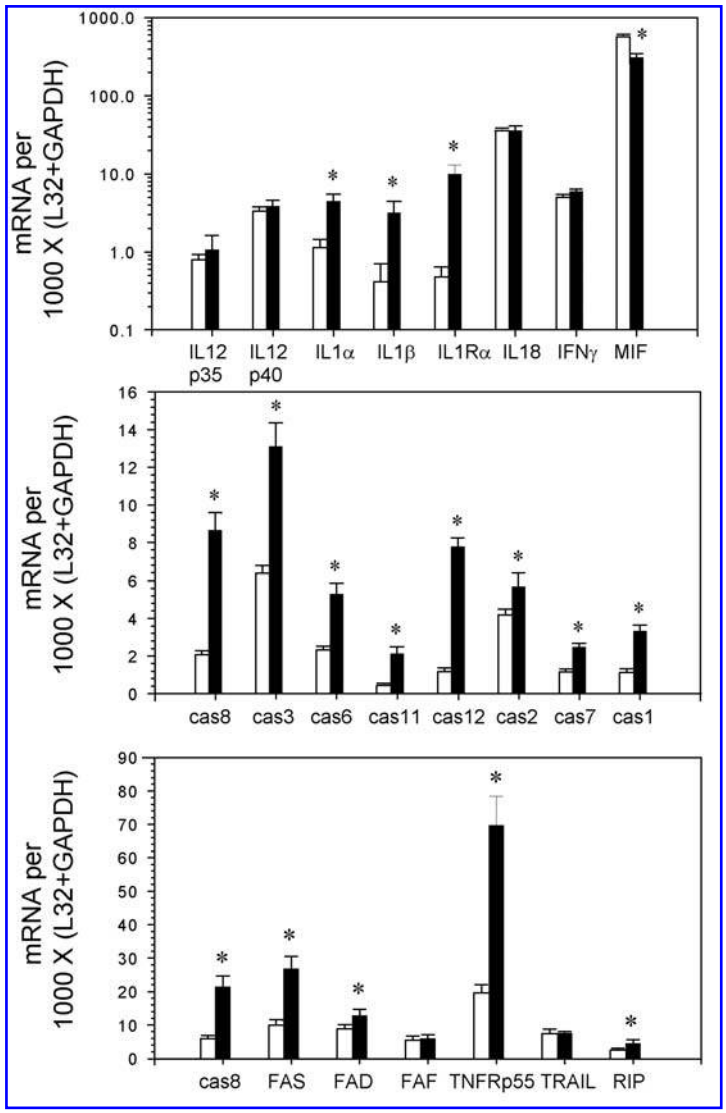

FIG. 5. RPA summary for cytokine transcription in spinal cord of 120-day-old G93A-SOD1 mice (*black bars*) compared with age-matched mice expressing similar levels of wild-type human SOD1 (*white bars*). The bar graphs indicate mean ± SD, $n = 7$ mice for each analyte.

paralysis in the G93A-SOD1 mouse (36, 37, 79). Contrastingly, T lymphocyte–derived cytokines (lymphokines) such as IL-2, IL-3, IL-4, IL-5, and IL-15 were expressed at lower levels, and these were only marginally altered in G93A-SOD1 mice.

Separate RPAs were performed to assess expression of apoptosis-associated genes at 120 and 80 days, representing symptomatic and late presymptomatic periods, respectively (36–39). All of the caspase mRNAs were increased at 120 days, including the TNFRI-associated caspase 8. Likewise, specific "death receptors" such as TNF receptor p55 (TNFRI) and FAS were unchanged or only slightly increased at 80 days but were strongly elevated by 120 days of age (36, 37) (Fig. 5). Thus, upregulation of proapoptotic genes generally follows cytokine changes but correlates with onset of bulk protein oxidation (discussed later) and onset of total hindlimb paralysis (Figs. 1 and 2). A particularly pronounced involvement of extrinsic death-signaling pathways activated by TNF superfamily members seems evident from these cumulative data and might suggest a link between chronic cytokine exposure and initiation of apoptotic programs. Figure 5 summarizes the principal significant findings gleaned from these RPA studies.

We have extended the RPA data by measuring a set of proinflammatory cytokines at the protein level, by using a multiplex antibody array technology (37). Findings from the antibody array pull-down study confirm the RPA results at the level of protein and indicate that multiple (but not all) proinflammatory cytokines, including TNF-α, are upregulated during symptomatic disease in G93A-SOD1 mice. Of possible interest, the chemokine (chemoattractant) protein RANTES was also significantly elevated in G93A-SOD1 mouse spinal cord (37).

It is likely important to note that cytokine upregulation occurs before frank motor neuron degeneration and therefore does not reflect localized reaction to dead and dying neural tissue. In very recent work from our laboratory, we found evidence that astroglial cells from G93A-SOD1 mice exist in a metastable state prone to cytokine elaboration as early as 7 days after birth. When primary astrocytes are cultured from

G93A-SOD1 mice, RPA analysis indicates that these cells express higher steady-state levels of TNF-α than do cells from nontransgenic littermates (38). Moreover, on stimulation with recombinant TNF-α protein or recombinant IFN-γ, G93A-SOD1 cells more aggressively upregulate *de novo* TNF-α transcription (38).

Cytokines and chemokines are not the only relevant paracrine factors that should be mentioned in discussions of neuroinflammation regarding the G93A-SOD1 mouse. Lipid-derived eicosanoids, including prostaglandin E_2 (PGE_2), a product of cyclooxygenase-II (COX-II) catalysis, are significantly increased approximately threefold in murine ALS spinal cord tissue (20) and in primary glia cultured from G93A-SOD1$^+$ neonatal mice (38). Leukotrienes derived by the action of arachidonic acid 5-lipoxygenase (5LOX) are likewise elevated in G93A-SOD1$^+$ primary glial cultures prepared from neonatal animals (38). Complementary work by MÖller's group (81) found similar *ex vivo* hyperactivity of adult (but not neonatal) G93A-SOD1 microglia, which produce quantitatively greater amounts of TNF-α in response to LPS stimulation than do nontransgenic microglia. It should be noted that both cyclooxygenase and lipoxygenase catalyze lipid peroxidation reactions, possibly leading to unstable lipid peroxyl intermediates if the normal enzymology becomes dysfunctional.

Thus, abundant evidence demonstrates the presence of a robust and broad-spectrum neuroinflammatory phenotype inherent to G93A-SOD1 transgenic CNS tissue. Very recent studies using primary glial cultures further suggest that non-neuronal CNS cells bearing mutant SOD1 transgenes exist in a metastable state prone to inflammatory signal transduction, even in very young presymptomatic animals.

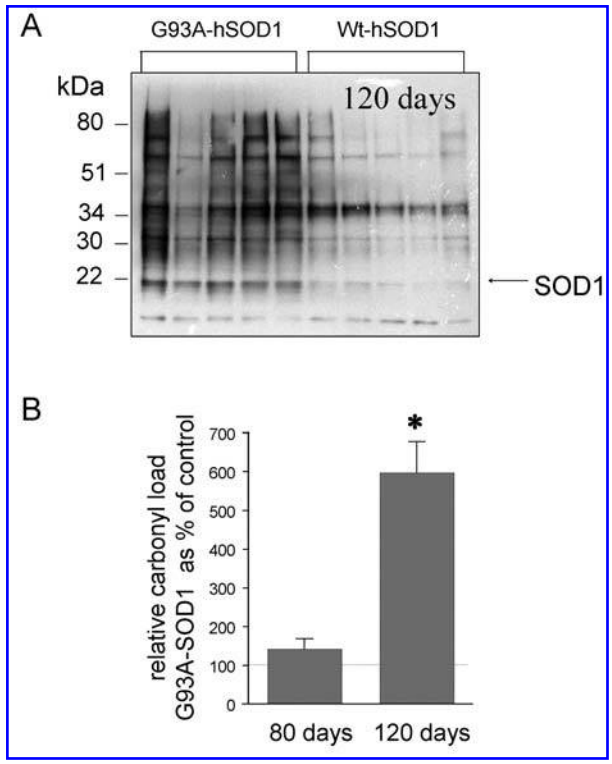

FIG. 6. (A) Protein carbonylation in postsymptomatic G93A-SOD1 mouse spinal cords as indicated by reaction of protein carbonyls with biotin hydrazide (36, 38). Each lane is derived from a separate mouse spinal cord. (**B**) Quantitation of protein carbonyl load in G93A-SOD1 mouse spinal cord at 80 and 120 days relative to matched nontransgenic littermates; bars indicate mean ± SD, $n = 5$ pairs at each age.

REDOX PERTURBATIONS IN MURINE AND HUMAN ALS

Protein oxidation and nitration have been reported both in murine ALS and human disease (4, 6, 10, 37, 38). To the extent that irreversible oxidative protein modifications have been measured in spinal tissue of SOD1-mutant mice, these modifications temporally correlate with the appearance of elevated cytokine production (4, 36, 37). By using a biotin hydrazide probe to covalently label protein carbonyls, we find a marginally significant 45% increase in total spinal cord protein carbonyl levels at 80 days of age in G93A-SOD1 mouse cord (Fig. 6). This is the timeframe in which RPA data first indicate transcriptional upregulation of TNF-α and interleukins (36, 37). Both protein carbonyl load and cytokine message increase throughout the progression phase of disease. Protein carbonylation measured immunologically with dinitrophenyl hydrazine labeling techniques revealed an almost exponential increase in these posttranslational modifications between 90 and 120 days in the G93A-SOD1 mouse (4).

The identity of individual oxidized proteins is the subject of ongoing investigations. SOD1 itself is a major carbonylated protein in SOD1 mutant spinal cord (4, 36), although wild-type human SOD1-overexpressing animals fail to carbonylate their SOD1 despite very similar magnitude of transgene expression (36). Kalyanaraman (86, 87) identified tryptophan-37 as a probable site of oxidation in mutant SOD1, yielding an *N*-formyl kynurenine derivative. Curiously SOD1 does not appear to be hyperoxidized in primary glial cultures from G93A-SOD1 mice, even on strong stimulation with inflammatory cytokines that heavily induce other protein carbonylations (discussed later). The meaning of this discordance remains unclear.

Recent proteomic studies have identified elevated oxidation in several proteins of the G93A-SOD1 spinal cord, including ubiquitin hydrolase and crystallin (60). Lipid oxidation products are likewise increased in ALS mouse spinal cord with hydroxynonenal (HNE)-protein adducts being found at elevated levels on certain major proteins, including heat-shock protein 70 (Hsp-70) (61). Further research likely will reveal additional oxidatively modified proteins relevant to ALS.

Neuroinflammation as a cause of oxidative stress: the importance of paracrine signaling molecules

For reasons discussed earlier, it seems likely that oxidative stress in murine ALS is not consequent to redox misbehavior of the mutant SOD1 *per se*. The robust inflammatory re-

sponse inherent to G93A-SOD1 central nervous system tissue provides an alternative explanation for oxidative stress in this animal model. It is now well established that certain cytokines, or aggregated immunoglobulin complexes, can trigger macrophages to produce copious quantities of reactive oxygen and nitrogen species through assembly of NADPH oxidase; induction of nitric oxide synthase (iNOS); and transcriptional upregulation of lipid-oxidizing enzymes such as inducible cyclooxygenase (COX-II). Microglia, the brain-resident macrophages, respond very similarly to peripheral macrophages with respect to their ROS- and RNS-generating capacity (15). Additionally, other cell types, including astrocytes, express nonphagocytic analogues of NADPH oxidase (NOX) (46).

Microglia respond to TNF-α, IL-6, IFN-γ, and aggregated IgG by producing nitric oxide and/or superoxide (15, 37, 39). We have explored this issue in some depth by using Walker EOC-20 microglia in cell-culture investigations. EOC-20 cells are a characterized, non–virus-transformed, CSF-1–dependent mouse microglia cell line that expresses IgG receptors FcγRI and II; Mac-1, Mac-2, Mac-3, CD45, CD80, and MHC-I constitutively and expresses MHC-II in response to IFN-γ (75, 79). EOC-20 cells therefore closely resemble macrophages and primary microglia. When TNF-α is added to EOC-20 cultures in a carefully titrated fashion so that marginal levels of cell stimulation are achieved, then the microglial NO_2^- production proves to be sensitively dependent on other ambient cytokines (79). Under these conditions, even small increments of IL-6 or IFN-γ can markedly magnify RNS output in a multiplicative rather than an additive fashion (37). Thus, even relatively modest twofold to fourfold increases in several synergistic cytokine species could disproportionately affect microglial activation. In other studies, we have investigated IgG ability to activate EOC-20 cells, because most studies of FcγR-mediated ROS/RNS formation have been conducted in monocyte or macrophage cell lines. As in the case of macrophage cells, IgG proved a particularly potent activator of RNS production by EOC-20 microglia, wherein IgG_{2a} is much more effective than IgG_1, which is a weak-affinity ligand for FcγRI (Fig. 7).

Data are beginning to emerge to suggest that G93A-SOD1$^+$ astrocytes or microglia are fundamentally more sensitive with respect to the manner in which these cell types respond to ambient paracrine signals such as inflammatory cytokines. MÖller and colleagues (81) cultured microglia from neonatal G93A-SOD1 mice or presymptomatic 60-day-old adults and stimulated the cells with bacterial LPS. No differences were observed between nontransgenic and G93A-SOD1 transgenic neonatal microglia in the basal or LPS-stimulated condition or between unstimulated adult cell cultures. Contrastingly, LPS-stimulated microglia cultured from 60-day-old G93A-SOD1 mice produced significantly more TNF-α and significantly less IL-6 than did matched nontransgenic cultures (81).

Our group has performed similar studies by using primary astroglial cultures from 7-day-old G93A-SOD1 mouse neonates. We find elevated basal production of TNF-α, iNOS, PGE_2, and LTB_4 in G93A-SOD1$^+$ cultures relative to cultures from nontransgenic littermates (38). Cultures from wild-type human SOD1-overexpressing animals did not reproduce

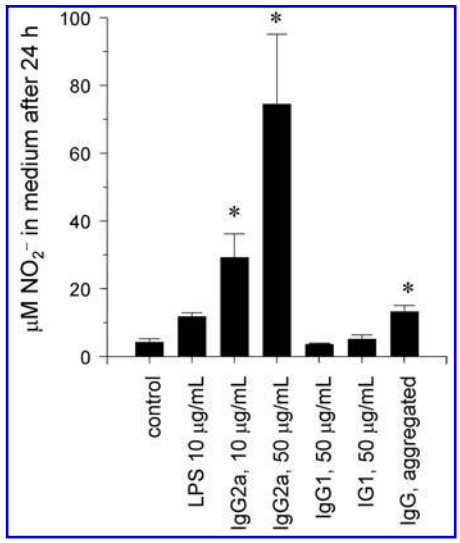

FIG. 7. RNS production by EOC-20 microglia after stimulation of FcγRI with IgG-2a (Lampire Laboratories, Pipersville, PA, U.S.A.), four to six experiments. *$p < 0.05$ by Student's t test. Note that uncomplexed IgG-1, a low-affinity ligand for FcγRI, does not activate EOC-20 cells.

eicosanoid alterations seen in G93A-SOD1 cells, thus largely alleviating the possibility of an artifact arising from general protein overexpression in the G93A-SOD1$^+$ cells (38). Stimulation of these cultures with TNF-α, alone or in combination with the potentiator IFN-γ, produces further elevations in iNOS, NO_2^- synthesis, and TNF-α expression. Most remarkably, protein carbonylation was exquisitely sensitive to presence of the G93A-SOD1 transgene (Fig. 8). Basal protein carbonylation was slightly greater in G93A-SOD1$^+$ astrocytes, whereas cytokine-stimulated protein carbonylation was elevated >100-fold in G93A-SOD1$^+$ cultures relative to identically treated, matched nontransgenic astrocyte cultures (Fig. 8) (38). Interestingly we were unable to detect carbonylated SOD1 in these hyperoxidized cultures (Fig. 8).

The most curious aspect of our studies with primary astrocytes is that none of the parameters found elevated in G93A-SOD1$^+$ cultures was elevated in the neonatal brains at the time of cell-culture isolation (38). Our interpretation of these data is that G93A-SOD1 expression renders glia into a metastable condition that is more prone to cytokine-stimulated ROS production and related gene-expression events, which is to say that signal-transduction dynamics in mutant SOD1-containing glial cells is fundamentally altered in a way that cannot be explained by general overexpression of SOD1 protein.

Oxidative stress as a possible cause of neuroinflammation: the importance of redox signal transduction

Reactive oxygen and nitrogen species have by now been thoroughly vindicated as authentic signal-transduction mediators. Mechanisms have been shown, and more mechanisms are being elucidated, through which specific oxidants propa-

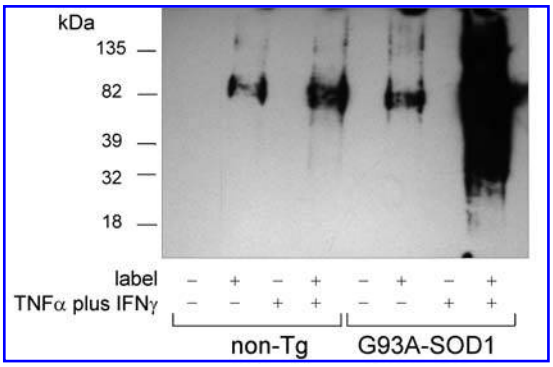

FIG. 8. Cytokine-stimulated cellular processes cause increased protein carbonylation in mutant SOD1-expressing glia. Astrocyte cultures were prepared from 1-week-old G93A-SOD1^{+} animals or nontransgenic littermates and challenged with TNF-α plus IFN-γ. After 48 h, cell lysates were probed for protein carbonyl derivatives by using the biotin hydrazide labeling technique (38). Samples indicated "±" label were, and were not (respectively) incubated with biotin hydrazide before polyacrylamide gel electrophoresis and streptavidin-peroxidase blotting procedures.

gate or modify signal transduction. For instance, NO was discovered as the agent that binds heme groups to activate critical effector enzymes such as guanylate cyclase, and other new targets are being discovered routinely. Hydrogen peroxide and nitric oxide interact with protein thiols to yield sulfenic acid derivatives and *S*-nitrosothiols, respectively, *en route* to reversibly glutathioned protein conjugates (reviewed in 24, 66). Protein thiol modification of protein tyrosine phosphatases (PTPs), in particular, seems to influence profoundly the gain of protein kinase amplification cascades by removing the reciprocal inhibition imposed by the phosphatases (24, 66). Detailed reviews of redox signal transduction can be found elsewhere (24, 66). Indirect perturbation of cellular redox balance with mitochondrial electron-transport chain inhibitors (*e.g.*, the complex III inhibitor antimycin-A) is also sufficient not only to activate protein kinase cascades in astrocytes, but also to produce *de novo* transcription of inflammatory cytokines including IL-6 and TNF-α, even in the absence of any other exogenous stimulus (24).

In theory, all that would be required for mutant SOD1 to influence redox signal transduction would be a positive interaction of the SOD1 with cellular ROS-generating systems or a negative interaction of the mutant protein with antioxidant systems. The resultingly perturbed redox balance might then increase the amplitude of intracellular signal created by cell-surface receptor engagement of proinflammatory cytokines, immunoglobulins, or other relevant paracrine ligands. As discussed earlier, mutant SOD1 may accumulate in mitochondrial intermembrane spaces, and mitochondrial alterations are prominent cytopathic events in both human and murine disease. One plausible mechanism for mutant SOD1-sensitized inflammatory signal transduction might then be manifest through functional mitochondrial alteration, leading to impaired electron transport and concomitantly increased superoxide or hydrogen peroxide leakage.

Another intriguing possibility is that cytochrome *c* released from ALS mitochondria could affect gene expression directly or indirectly. Clearly no frank apoptosis happens in ALS motoneurons within the period of 30–90 days, despite detectable Cyt-c leakage (28). Sobue's group (70) published complementary data suggesting that mitochondria-targeted mutant SOD1, but not wild-type SOD1, is capable of promoting Cyt-c release into the cytosol.

An open question regards what, if anything, this extramitochondrial Cyt-c might do besides directly initiating cell-death programs. Cyt-c is a redox-active heme protein with some peroxidase activity that can increase 1,000-fold on proteolytic cleavage that generates microperoxidase, an 8- to 12-amino acid polypeptide retaining ligated heme (5). Antibodies that recognize full-length Cyt-c would not recognize truncated microperoxidase, a fact that may require consideration in future studies of mitochondrial alteration in ALS. Cyt-c has been shown to bind certain transcription factors such as AP2 *in vitro* (32). In the case of AP2 binding, Cyt-c apparently oxidizes the transcription factor and increases transcription-factor affinity for its DNA consensus sequence (32). The COX-II promoter contains possible AP2-binding sites (71), and AP2 synergizes with other factors to promote COX-II expression in some cell lines (45). Thus, important, unappreciated roles may be played by extramitochondrial Cyt-c in altering redox signal transduction within glial cells during key phases of ALS pathogeny.

The preceding discussion and literature review suggests that neuroinflammation may be considered as something of a vicious cycle wherein oxidative stress (impaired or perhaps exacerbated redox signal transduction) and increased proinflammatory paracrine factor synthesis are antipodes of the positive-feedback loop. Marginally increased redox signal transduction might increase the sensitivity of cytokine gene expression, for instance, or might encourage expression of inducible COX and NOS enzymes. The resulting, slight increase in ambient cytokines or inflammatory cell-surface receptors would then increase the likelihood of further oxidative stress through established enzyme-dependent pathways, including NADPH oxidase. Figure 9 attempts to illustrate these ideas schematically in the context of a relevant astroglial or microglial cell. Such a model is not complete and cannot fully explain ALS pathogenesis; however, this general model might explain some aspects of disease and might begin to predict productive avenues for researching new therapeutic strategies against motor neuron degeneration.

PROGRESS TOWARD ALS THERAPY: SUGGESTIONS FROM NEUROINFLAMMATION THEORY

Classic nonsteroidal antiinflammatory agents target prostaglandin formation by constitutive or inducible cyclooxygenase. Clearly evidence exists of COX involvement with ALS (20, 38); however, COX is only one of many proinflammatory components that are upregulated in rodent models of disease. Other CNS-available therapeutic modalities that target alternative branches of arachidonate metabolism, or other inde-

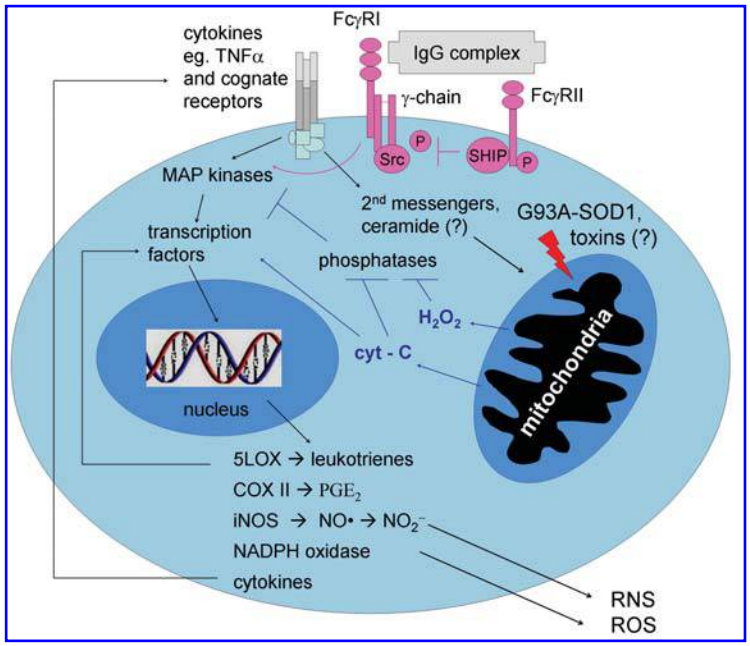

FIG. 9. Schematic illustrating several major motifs of neuroinflammatory signal transduction that have been implicated in murine models of heritable, mutant SOD1-induced, amyotrophic lateral sclerosis. This figure is meant to exemplify either an astrocyte or microglia, although similar transduction pathways are likely relevant to neurons as well. TNF-α is used as an archetypal cytokine that is particularly pertinent to ALS; however, other pro- and antiinflammatory cytokines plus various chemokines and eicosanoids must be considered as paracrine factors that activate cell-surface receptors on either neurons or glia (or both). Special attention in this review is given to IgG and IgG receptors FcγRI (stimulatory) and FcγRII (inhibitory), both of which have been implicated in G93A-SOD1 mouse models of ALS. In this schematic, arrows (→) indicate stimulatory signals, whereas brakes ( ) indicate inhibitory components. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars.)

pendent aspects of glial inflammation, might prove valuable. Toward this end, data obtained from murine ALS models might help inform the decision-making process of translational and clinical scientists. Thus far, several compounds with possible antineuroinflammatory properties have proved modestly efficacious against the G93A-SOD1 mouse model of ALS. Minocycline, a tetracycline derivative with macrophage- and microglia-suppressing effects, has shown some modest efficacy against murine ALS (88). Celecoxib, a COX-II–selective inhibitor, significantly slows disease in murine models (20), although human trials have thus far proven unsuccessful. Our group has found that nordihydroguaiaretic acid (NDGA), an arachidonate 5-lipoxygenase inhibitor that potently inhibits TNF-α–stimulated EOC-20 microglial activation (79), significantly extends the life span and slows paralytic disease in G93A-SOD1 mice when administered orally beginning at 90 days of age. In this case, NDGA was selected by screening hundreds of prospective drugs for the ability to antagonize microglia *in vitro*. Most recently, Beal and colleagues (44) reported that thalidomide, a much-studied but teratogenic drug that inhibits TNF-α production, significantly protects against murine ALS.

Although preliminary, these findings encourage further consideration of neuroinflammation as one guiding principle to aid in the selection of lead molecular structures *en route* to optimized therapeutic agents for ALS and other neurodegenerative diseases associated with strong neuroinflammatory components.

CONCLUSIONS

Several limited conclusions can be drawn from the preceding review. It is clear that neuroinflammation is a prominent, readily studied phenomenon in mouse models and likely the newer rat model for mutant SOD1-associated, heritable amyotrophic lateral sclerosis. It is not clear whether neuroinflammation in the rodent models is sufficient to cause motor neuron death or even contribute significantly to motor neuron dysfunction, as opposed to being an especially curious epiphenomenon. It may be the case that certain aspects of neuroinflammation are neurodestructive, whereas other aspects are neuroprotective, so that the balance of competing phenomena helps determine the rate of disease progression. These questions demand more attention in future studies of the G93A-SOD1 and related transgenic systems. With these qualifications, neuroinflammation in G93A-SOD1 mice does seem intimately and mechanistically linked to protein oxidative damage, as clearly evidenced from recent studies of SOD1-mutant glial cell cultures in which ROS/RNS generation and protein oxidation are exquisitely sensitive to stimulation by ambient proinflammatory cytokines.

Furthermore, even if neuroinflammation is a significant contributor to pathobiology in murine ALS, it remains unclear how completely available animal models accurately reproduce the human situation. Studies of human ALS tissue have been fewer and more equivocal with regard to the nature and extent of neuroinflammatory processes at work. Certain plausibly pathologic components of neuroinflammation seem to be shared between G93A-SOD1 mice and human sporadic ALS. These include involvement of the tumor necrosis factor superfamily of paracrine mediators; activation of IgG signaling pathways in the CNS (the meaning of which has been remarkably underexplored and remains an important topic for future scientific scrutiny); and increased production of eicosanoids derived from enzyme-mediated oxidations of polyunsaturated fatty acids.

SOD1-mutant animals have proven a robust system for the study of innate immune reactions of the neuroinflammatory type and also for studies of aberrant redox biochemistries. This work has only just begun and should continue for some time. The availability of specific transgenic and knockout an-

imals, and new emerging technologies for RNA silencing, will allow more-refined molecular dissection of neuroinflammatory pathways at work in SOD1-mutant animals. Care must be taken in such future studies to avoid artifacts from strain background mixing, which can be circumvented by using SOD1-mutant animals and complementary genetically modified animals all maintained on a uniform C57/B6 background. Potential pleiotropic effects of target proteins (*e.g.*, developmental necessity for TNF-α during gestation) also require consideration in attempted knockout-rescue experiments so that age-dependent conditional knockdown studies might be devised. Nonetheless, the technologies currently exist to probe and understand individual inflammatory pathways at work in murine ALS. Given time and sufficient research effort, we can hope that consideration of these pathways will identify exploitable molecular targets and strategies to treat ALS and other, currently intractable neurodegenerative diseases.

ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; AP2, activator protein 2; CNS, central nervous system; COX-II, inducible cyclooxygenase; Cyt-c, cytochrome *c*; FALS, familial amyotrophic lateral sclerosis; FcγRI/FcγRII/FcγRIII, receptors for IgG; HSP-70, heat-shock protein 70; IFN-γ, interferon gamma; IL-1, interleukin 1; IL-6, interleukin 6; IL1-RA, interleukin 1 receptor antagonist; IgG, immunoglobulin G; 5-LOX, arachidonic acid 5-lipoxygenase; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; MCP-1, macrophage chemoattractant protein-1; M-CSF, monocyte colony-stimulating factor; MHC, major histocompatibility complex; NADPH, nicotinamide adenine dinucleotide phosphate; NDGA, *nor*-dihydroguaiaretic acid; NOS, nitric oxide synthase; NOX, nonphagocytic NADPH oxidase; PGE_2, prostaglandin E_2; PTP, protein tyrosine phosphatase; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPA, ribonuclease protection assay; SALS, sporadic amyotrophic lateral sclerosis; SOD1, cytosolic Cu-Zn superoxide dismutase; TNF-α, tumor necrosis factor alpha.

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