Phenotypically aberrant astrocytes that promote motoneuron damage in a model of inherited amyotrophic lateral sclerosis

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Motoneuron loss and reactive astrogliosis are pathological hallmarks of amyotrophic lateral sclerosis (ALS), a paralytic neurodegenerative disease that can be triggered by mutations in Cu-Zn superoxide dismutase (SOD1). Dysfunctional astrocytes contribute to ALS pathogenesis, inducing motoneuron damage and accelerating disease progression. However, it is unknown whether ALS progression is associated with the appearance of a specific astrocitic phenotype with neurotoxic potential. Here, we report the isolation of astrocytes with aberrant phenotype (referred as “AbA cells”) from primary spinal cord cultures of symptomatic rats expressing the SOD1<sup>G93A</sup> mutation. Isolation was based on AbA cells’ marked proliferative capacity and lack of replicative senescence, which allowed oligoclonal cell expansion for 1 y. AbA cells displayed astrocitary markers including glial fibrillary acidic protein, S100β protein, glutamine synthase, and connexin 43 but lacked glutamate transporter 1 and the glial progenitor marker NG2 glycoprotein. Notably, AbA cells secreted soluble factors that induced motoneuron death with a 10-fold higher potency than neonatal SOD1<sup>G93A</sup> astrocytes. AbA-like aberrant astrocytes expressing S100β and connexin 43 but lacking NG2 were identified in nearby motoneurons, and their number increased sharply after disease onset. Thus, AbA cells appear to be an as-yet-unknown astrocyte population arising during ALS progression with unprecedented proliferative and neurotoxic capacity and may be potential cellular targets for slowing ALS progression.

Results

Establishment of Astrocyte Cultures from Symptomatic SOD1<sup>G93A</sup> Rats. To isolate glial cell populations occurring during the symptomatic stage of ALS, we established primary cultures from spinal cord of symptomatic 175-d-old Tg SOD1<sup>G93A</sup> rats. Identical cultures from non-Tg littermates prepared in the same way yielded only a few cells at day 2 in vitro that proliferated slowly in the following days but failed to reach confluence or survive subsequent passages (Fig. 1, Insets). In contrast, cultures from Tg rats yielded numerous cells that proliferated rapidly at day 7 in vitro (Fig. 1) and formed clusters of elongated flat cells resembling astrocytes and often associated with numerous ionized calcium-binding adaptor molecule 1 (Iba1)-positive microglial cells (arrows in Fig. 1, and Fig. S1C). At day 10 in vitro, a 3- to 4-mm layer of agarose was polymerized on top of the cell layer.

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AbA cells were generated systematically from more than 10 independent cultures prepared from different symptomatic Tg rats, suggesting that they represent a common cell type resident in the degenerating spinal cord during the symptomatic phase of the disease.

Expression of Astrocytic Markers in AbA Cells. We next assessed the expression of astrocytic markers in AbA cell cultures from passages 8–15. Because we were unable to grow and passage astrocytes from non-Tg adult rats, the phenotypic features of AbA cells were compared with primary spinal cord astrocytes prepared from neonatal SOD1<sup>G93A</sup> rats and non-Tg littermates. Among the antigenic markers listed in Table S1, we found that both AbA cells and neonatal astrocytes expressed most of the typical astrocytic markers such as GFAP, vimentin, S100β, connexin 43 (Cx43), and glutamine synthase, but GLT1 protein was not detected in AbA cells. NG2 glycoprotein was not expressed in AbA cells immediately after culture establishment, but it increased gradually, beginning after passage 4 (Fig. S1B). AbA cells were negative for A2B5, oligodendrocyte transcription factor 2, and CD68 antigen clone (ED1), which are expressed by glial precursors, oligodendrocyte progenitor cells, and phagocytic microglia, respectively (Table S1).

AbA cells displayed weak and diffuse perinuclear GFAP labeling and intense staining for S100β, in contrast with the filamentous GFAP and low S100β expression in neonatal astrocytes (Fig. 2A). GFAP levels in AbA cells were decreased by 60% in comparison with neonatal astrocytes, as estimated by Western blotting (Fig. 2B). AbA cells displayed processes and increased GFAP staining when challenged with forskolin (10 μM; Fig. S1D), a recognized stimulus that promotes astrocyte differentiation in vitro (24). AbA cells expressed Cx43 in a cytoplasmic and patchy surface distribution (Fig. 2A) at levels increased fivefold in comparison with control astrocytes (Fig. 2B). The expression of glutamine synthase (Fig. 2B and Fig. S1D) and vimentin (Fig. S1D) in AbA cells was comparable to that found in neonatal SOD<sup>G93A</sup> astrocytes.

**AbA Cell Proliferation.** The growth of AbA cells through passage 10 was faster than that of Tg and non-Tg neonatal astrocytes (Fig. 2C). The doubling times, calculated during the linear phase of the growth curve, were 30 h for AbA cells, 52 h for non-Tg astrocytes, and 49 h for Tg astrocytes. AbA cell numbers con-

![Fig. 2](image-url)
continued increasing after reaching confluence at day 5 in vitro, suggesting a defect in contact inhibition.

**AbA Cells Specifically Induced Motoneuron Death.** Because astrocytes carrying the SOD1<sup>G93A</sup> mutation have been shown specifically to induce motoneuron death (10–12), we assessed the neurotoxic potential of AbA cells by plating embryonic motoneurons (25) on top of confluent AbA or neonatal astrocyte monolayers. The survival rate after 2 d was <10% for motoneurons in cocultures with AbA cells but was 60% for neurons cocultured with Tg cells and was 100% for neurons cultured with non-Tg neonatal astrocytes (Fig. 3A). These data suggest that AbA cells exert a marked nonpermissive environment for motoneuron growth and differentiation.

We next investigated whether AbA cell toxicity was mediated by soluble factors secreted to the conditioned media (CM), as previously described for mutant primary astrocytes (12). Increasing dilutions of CM from AbA cells or neonatal astrocyte cultures having similar cell number were added to embryonic motoneuron cultures maintained with glial cell-derived neurotrophic factor (GDNF) as the trophic factor. As expected, CM from neonatal non-Tg astrocytes did not cause motoneuron death (100% survival in Fig. 3B). In contrast, after exposure to AbA CM in a range of dilutions from 1:10 to 1:1,000, the number of motoneurons was significantly lower than the number surviving after addition of CM from neonatal Tg astrocytes, which reduced motoneuron survival significantly only at dilutions up to 1:100. Thus, the neurotoxic potential of AbA CM is at least 10-fold greater than that of Tg astrocyte CM (Fig. 3B). Notably, the neurotoxicity of AbA CM was specific to motoneurons, because even a 1:10 dilution failed to kill primary cultures of embryonic hippocampal neurons (Fig. 3C).

**Identification of AbA-Like Cells in the Degenerating Spinal Cord.** We used immunohistochemistry to S100β/Cx43 to determine whether AbA-like cells were present in the degenerating spinal cord of SOD1<sup>G93A</sup> rats. S100β staining was low or moderate in non-Tg and Tg asymptomatic rats. In contrast, S100β increased dramatically during the symptomatic stage of Tg rats, being localized in the nuclei and cytoplasm in a population of hypertrophic astrocytes that expressed GFAP restricted to cell bodies and proximal processes. Typically, these cells were observed in the ventral spinal cord, near damaged motoneurons, and at the boundary between gray and white matter. Cx43 staining also increased dramatically in symptomatic rats and colocalized with S100β hypertrophic astrocytes (Fig. 4). Such astrocytes appeared at the time of disease onset, and their number increased sharply at disease end stage (Fig. 4B). In the ventral horn of symptomatic rats, S100β did not colocalize with NG2 oligodendrocyte precursors previously described in ALS mice (Fig. 4C; Manders coefficient ≤ 0.02), suggesting that S100β cells constitute a different cell population. Furthermore, S100β<sup>+</sup> or Cx43<sup>+</sup> AbA-like cells surrounding motoneurons were labeled with the proliferation markers Ki67 or BrdU in animals systemically injected with the nucleotide (Fig. S2C). The number of Ki67<sup>+</sup> proliferating AbA-like cells in the ventral cord region enriched in motoneurons represented 20 ± 5% of cells, compared with 33 ± 8% proliferating NG2 cells and 43 ± 10% microglia (Fig. S2B).

**Discussion.**

Neuronal degeneration in ALS begins as a focal process that spreads contiguously through the upper and lower motoneurons (26), suggesting an acquired pathogenic mechanism in which motoneuron pathology and inflammation actively propagate in the CNS. Here we report the isolation of a type of astrocyte with aberrant phenotypic features (AbA cells) and an unprecedented neurotoxicity of AbA cells in vitro from symptomatic SOD1<sup>G93A</sup> rats. Notably, AbA cells exhibit a distinctive pattern of astrocytic markers with an increased proliferation rate and a lack of replicative senescence. Proliferating AbA-like astrocytes were localized near motoneurons in the spinal cord of symptomatic SOD1<sup>G93A</sup> rats, suggesting a link between the appearance of pathogenic AbA cells and the rapid progression of paralysis characteristic of the SOD1<sup>G93A</sup> rat model.

The proliferative potential of AbA cells after isolation from the Tg spinal cord was strong enough to allow their oligoclonal expansion in conditions where age-matched non-Tg rats yielded few cells with limited growth potential. The AbA proliferation rate was almost twice that of the neonatal astrocytes but still was far below that of the C6 astrocyte cell line (27). Thus, AbA cells do not appear to be fully transformed cells, even though they do not follow replicative senescence. Cultured AbA cells are almost undistinguishable morphologically from primary neonatal astrocytes and exhibit a set of distinctive antigenic markers of undifferentiated astrocytes including high S100β and Cx43 expression and low levels of nonfilamentous GFAP. As a prototypic subunit of the calcium-binding S100 proteins, S100β is known to exert paracrine effects in astrocytes that contribute to proliferation, migration, differentiation, and neurotoxicity (28–30). Intra-cellular S100β can interact with GFAP monomers to prevent their assembly into filaments (31), possibly contributing to the
diffuse GFAP distribution in AbA cells. Forskolin, which induces astrocytic process growth and differentiation (24), also increased process growth and GFAP expression in AbA cells, further indicating their astrocytic phenotype.

Another distinctive marker of AbA cells is the high expression of the gap junction protein Cx43, which also is found in cultured neonatal astrocytes (32) and is known to modulate their proliferation, migration, and differentiation (33). Cx43 also can form hemichannels opened to the extracellular space in inflammatory astrocytes (34, 35), which can release extracellular ATP (34, 36). Thus, the high levels of Cx43 in AbA cells may explain their potential to trigger glial activation and excitotoxic degeneration of motoneurons (37). The blunted levels of astroglial glutamate transporter GLT1 protein in AbA cells suggest that these cells recapitulate a defect in GLT1 expression previously described in ALS patients (38) and SOD1G93A rats (3). Because GLT1 is expressed in differentiated astrocyte endings to uptake synaptic glutamate, accumulation of AbA cells around motoneurons may promote further excitotoxic damage in vivo.

Furthermore, AbA cells do not appear to be derived from NG2 oligodendrocyte progenitors that recently have been reported to proliferate in the ALS spinal cord after disease onset (16, 17). AbA-like cells in the spinal cord of symptomatic rats are NG2- and display a morphology and location distinct from that of typical NG2 cells. In addition, AbA cells were NG2- when first established in culture, and the glycoprotein was expressed only after more than four passages. Characteristic AbA cells coexisted with and were easily distinguishable from NG2+ cells when primary cultures of the spinal cord were prepared in a defined medium that favors oligodendrocyte differentiation.

AbA cells represent a population of glial cells with undifferentiated features. Thus, their generation in the spinal cord could be associated with the overt inflammatory microenvironment that accompanies motoneuron loss, particularly in the SOD1G93A animal models (39). Such a milieu may promote the recruitment and phenotypic transition of glial cells or precursors (40), leading to the generation of AbA cells. A similar prolife-rative and immature phenotype of astrocytes can be found following defective function in paired box 6 (Pax6) transcription factor (41) and in pathological conditions leading to aberrant specification of glial precursors (42).

Previous studies have shown that astrocytes bearing the SOD1G93A mutation induce apoptosis of motoneurons both in coculture conditions and through soluble factors found in the culture media (10–12, 19, 20), whereas non-Tg astrocytes provided excellent trophic support. However, we show here that AbA cells display an unprecedented toxicity to motoneurons that greatly exceeds that of neonatal Tg astrocytes expressing mutant SOD1. The complete failure of motoneurons to survive when plated on a confluent layer of AbA cells suggests that AbA cells create a nonpermissive microenvironment for motoneuron growth. This effect could be mediated at least in part by the accumulation of specific extracellular matrix proteins that are known to be up-regulated in ALS rats (43). In addition, the AbA cell CM were >10-fold more potent than neonatal Tg astrocytes in inducing motoneuron death. Because AbA cell CM were applied in the presence of GDNF, which supports motoneuron survival, it is unlikely the toxicity is caused solely by decreased trophic activity. Rather, AbA cells appear to be a subclass of astrocytes producing active soluble factors that kill motoneurons. Although the mechanism of AbA neurotoxicity is under active investigation, the possibility exists that AbA cells produce cytokines, excitotoxins, or trophic factors such as nerve-growth factor that may kill motoneurons specifically (8).

Hypertrophic AbA-like cells strongly expressing S100beta and Cx43 were identified in the degenerating spinal cord, suggesting a common phenotype with AbA cells isolated in culture. Moreover, BrdU+ and Ki67+ AbA-like cells were found systematically in close contact with degenerating motoneurons, suggesting that they proliferate in the ventral horn as the rat becomes paralytic. Evidence for the occurrence of astrocytic-like BrdU+ nuclei
coexisting with dividing NG2 cells was provided previously by Kang et al. (17) in SOD1<sub>G93A</sub> mouse spinal cord. Although AbA-like cells represented only about 20% of the proliferating glial cells in the ventral cord gray matter, they were more closely associated with degenerating motoneurons than were NG2<sup>+</sup> cells and frequently were associated with proliferating microglia. Perineuronal S100<sup>β</sup> astrocytes were described previously in ALS patients (44), suggesting that AbA cells also may occur in human terminal disease. S100<sup>β</sup> can form heterodimers with S100A6, which is up-regulated specifically in reactive astrocytes occurring in ALS patients and mutant SOD1 Tg animals (45). The increased expression of Cx43 in AbA cells has not been reported in ALS, neurogenic activation of spinal cord astrocytes is known to induce Cx43 (46) following axotomy and spinal cord injury (47). The fact that blocking Cx43 in spinal cord injury improved recovery (48) anticipates a pathogenic role of Cx43 up-regulation in ALS.

In conclusion, the present study addressed one key question about ALS pathogenesis: the identification and isolation of an astrocyte population with the potential to mediate motoneuron disease. Such astrocytes were abundant in the symptomatic phase of disease and typically localized close to motoneurons, suggesting a link between the emergence of pathogenic AbA cells and the rapidly progressing neurodegeneration characteristic of the SOD1<sub>G93A</sub> rat model. Thus, AbA cells represent an intriguing cell target for further understanding the pathogenesis of neurodegenerative diseases.

Materials and Methods

Details for materials and methods used in this study are provided in SI Materials and Methods. Animals. Male hemizygous N Tac-SD-Tg(SOD<sub>G93A</sub>)26H rats (Taconic), originally developed by Howland, et al. (3), were bred locally as outbred Sprague-Dawley background. The onset of symptomatic disease (~160 d) and lifespan (180 d) in our colony were delayed considerably compared with the original report (3).

Establishment of AbA Cell Cultures. AbA cells were obtained from adult spinal cord of symptomatic SOD1<sub>G93A</sub> rats (175 d) according to the procedures described by Saneto and De Vellis (49) with minor modifications (25). Adult aggregates of non-Tg astrocytes were used as controls. Briefly, animals were killed by deeply anesthesia, and spinal cord was dissected on ice. After the meninges were removed carefully, spinal cord was chopped finely with a scalpel, and the resultant pellet was resuspended in DMEM/10% (vol/vol) FBS and plated at a density of 1 × 10<sup>6</sup> cells per 25-cm<sup>2</sup> culture flask. After confluence, each flask yielded up to 2 × 10<sup>6</sup> cells, and 1 wk after seeding each bottle was divided in two bottles. AbA cells were cultured in the same medium during the entire procedure.

In some experiments, primary cultures from spinal cord of symptomatic Tg rats were plated on 0.1 mg/mL polylysine-covered plates and maintained in oligodendrocyte-defined medium (Neurobasal medium supplemented with B-27) for 2 d (23).

Primary Cell Cultures. Heterozygous Tg and non-Tg astrocytes were prepared from spinal cords of 1-d-old pups according to the methods described by Saneto and De Vellis (49) with minor modifications (25). Motoneuron cultures were prepared from embryonic day 15 wild-type rat spinal cords and purified by immunopanning (25). Motoneurons were seeded on polylysine-laminin-coated substrate and maintained in Neurobasal medium supplemented with GDNF (1 ng/mL) (Sigma). After 24 h in vitro, motoneurons were treated with dilutions of CM. Survival was assessed after 48 h. For preparation of CM, confluent neonatal astrocyte and AbA monolayers were incubated in complete L15 medium for 24 h. Respective supernatants were centrifuged at 1,000 × g for 15 min and were applied immediately to motoneuron cultures. For all the experiments, motoneurons were plated on confluent AbA or neonatal astrocyte monolayers and were maintained for 48 h in complete L15 medium supplemented as previously described (25). Hippocampal neuronal cultures were obtained from embryonic day18 embryos as described in SI Materials and Methods.

Western Blots. Protocols for Western blotting are described in SI Materials and Methods.

Assessment of Cell Proliferation. Cell proliferation was assessed as described in SI Materials and Methods by counting the number of viable nuclei.

Immunolabeling. Methanol-fixed cultured cells or paraformaldehyde perfusion-fixed floating spinal cord sections were processed for immunocytochemistry or immunohistochemistry as described in SI Materials and Methods and Table S1. Quantification of AbA-like cells in the ventral horn of spinal cord sections was done by direct counting of hypertrophic cells that surround motoneurons and exhibit cytoplasmic S100<sup>β</sup>.

Statistical Analysis. Statistical studies were performed using statistical tools of Origin 8.0. Descriptive statistics were used for each group, and one-way ANOVA, followed by Scheffé post hoc comparison if necessary, was used among groups. All experiments were performed in duplicate or triplicate and were replicated at least three times. All results are presented as mean ± SD. P < 0.05 was considered significant.

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