

Advanced Glycation Endproducts in Neurofilament Conglomeration of Motoneurons in Familial and Sporadic Amyotrophic Lateral Sclerosis

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Abstract

Background: Massive neurofilament conglomeration in motor neurons has been described to occur in the early stages of both familial and sporadic amyotrophic lateral sclerosis (ALS). Previously, neurofilament conglomerates were immunolabeled for both superoxide dismutase (SOD1) and nitrotyrosine, suggesting the potential for oxidative nitration damage to neurofilament protein by peroxynitrite. Long-lived neurofilaments may also undergo modification by advanced glycation endproducts (AGEs) with concomitant generation of free radicals, including superoxide. This radical species may then react with nitric oxide to form the potent oxidant, peroxynitrite, which in turn can nitrate neurofilament protein. Such a glycosylated and nitrated neurofilament protein may become resistant to proteolytic systems, forming high-molecular-weight protein complexes and cytotoxic, neuronal inclusions.

Materials and Methods: Paraffin sections containing both neurofilament conglomerates and neuronal inclusions were obtained from patients with sporadic ($n = 5$)

and familial ($n = 2$) ALS and were probed with specific antibodies directed against the AGEs cypentodine/piperidine-enolone, arginine-lysine imidazole, pentosidine, and pyrroline.

Results: Neurofilament conglomerates, but not neuronal inclusions, were intensely immunolabeled with each of the anti-AGE antibodies tested. The immunoreactivity was selective for neurofilament conglomerates and suggested that AGEs may form inter- or intramolecular cross-links in neurofilament proteins.

Conclusions: These data support the hypothesis that AGE formation affects neurofilament proteins in vivo and is associated with the concomitant induction of SOD1 and protein nitration in neurofilament conglomerates. AGE formation in neurofilament protein may not only cause covalent cross-linking but also generate superoxide and block nitric oxide-mediated responses, thereby perpetuating neuronal toxicity in patients with ALS.

Introduction

The seminal pathologic feature of early amyotrophic lateral sclerosis (ALS), a disease of neuro-

filament-rich neurons, is massive neurofilament conglomeration in motor neurons (1,2). The most extreme changes include the formation of axonal spheroids in lower motor neurons and conglomerates in upper motor neurons (3). Both familial and sporadic ALS show these changes on histopathological examination (2,4,5). The study of neurofilament conglomeration is critical for

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Table 1. Antibody immunoreactivities

Antibody	Source	mAb/pAb	Dilution	Axs	Cgl	MN	HI
Cy/Pi ^a	Makita et al. (30)	mAb	1:24,000	+3	+4	+1	—
Arg-Lys Imi ^a	Al-Abed and Bucala (31)	pAb	1:900	+3	+3	+1	—
Pentosidine	Smith et al. (35)	pAb	1:500	+2	+2	+1	—
Pyrraline	Smith et al. (35)	mAb	1:300	+3	+2	+1	—
SOD1	Sigma	mAb	1:500	+3	+3	+1	+1
Ubiquitin	DAKO	pAb	1:50	+2	+1	—	+4
NF-p	SMI	mAb	1:100	+4	+4	—	—
NF-np	SMI	mAb	1:100	+3	+4	+2	+1
NF-L-np	Zymed	mAb	1:50	+3	+4	+1	—
b-NOS	Affinity	pAb	1:200	+1	+2	—	+1
Nitrotyrosine	Beckman et al. (9)	mAb	1:40	+1	+2	+1	—

Axs, axonal spheroids; Cgl, conglomerates; MN, motor neurons; HI, hyaline inclusions; mAb, monoclonal antibody; pAb, polyclonal antibody; NF-p, phosphorylated neurofilament; NF-np, nonphosphorylated neurofilament; NF-L-np, nonphosphorylated neurofilament light-subunit; b-NOS, brain nitric oxide synthase.

^aThe anti-cypentodine/piperidine-enolone (Cy/Pi) and anti-arginine-lysine imidazole (Arg-Lys Imi) do not react significantly with pyrraline, pentosidine, or carboxymethyl-lysine.

understanding the pathogenesis of ALS, as various ubiquitin(+)/SOD1(+) neuronal inclusions, such as hyaline, Lewy body-like, or skein-like inclusions, may derive from neurofilament conglomerates (4–7). The discovery of an SOD1 gene mutation in a small fraction of familial ALS patients (8) has compelled us to examine potential oxidative damage of motor neuron proteins in ALS and to correlate this with reactive oxidative species such as superoxide and nitric oxide. Nitric oxide is believed to be the only biologically derived molecule that can supersede SOD1 for capture of superoxide, and both tyrosine nitration and metabolites of the nitric oxide/cGMP cascades have been demonstrated in SOD1-positive neurofilament conglomerates (9–12).

Neurofilament aggregation is a chronic process that may also involve the time-dependent cross-linking of neurofilament protein by advanced glycation endproducts (AGEs). In macromolecules such as low-density lipoprotein, AGE formation has been associated with oxidative modification and free radical production in vitro (13–15). Because the neuronal inclusions in ALS have been reported to be immunoreactive for SOD1 (16–22), nitrotyrosine (11,12,23–26), and ubiquitin (5,6,27,28), we considered the possibility that AGE formation in neurofilament conglomerates or axonal spheroids may play a role in the formation of protein complexes and the resultant neurotoxicity that characterizes ALS.

Materials and Methods

Brain and spinal cord specimens were obtained from seven ALS patients; (five had a history of sporadic ALS with prominent axonal spheroids and two had familial ALS with scattered conglomerates in the motor cortex) and one normal control. Four segments were obtained from the lumbar spinal cord and the brain sections were obtained from the motor cortex. Tissue was fixed in 10% formaldehyde, embedded in paraffin, cut at 7 μ m thickness, and stained with hematoxylin and eosin for confirmation of the presence of neurofilament conglomerates. Immunohistochemical staining was performed by a Ventana automated system (Ventana, Tucson, AZ), applying avidin-biotin complex with 3,3'-diaminobenzidine (DAB) as the chromogen. Paraffin sections were immunostained with antibodies against the AGEs [cypentodine/piperidine-enolone (29), arginine-lysine imidazole (30,31), pentosidine, and pyrraline (32)]. The sources and working dilutions of the primary antibodies against AGEs and other antigens are summarized in Table 1. Immunostaining for SOD1, ubiquitin, and both phosphorylated and nonphosphorylated neurofilament subunits (light, NF-L; medium, NF-M; heavy, NF-H) for confirmation of neurofilament conglomeration was performed as previously reported (11,12). Preabsorption testing for each of the anti-AGE antibodies was per-

formed as previously described (32–35). Antibody to AGE was incubated with respective antigen, AGE-BSA (bovine serum albumin) for monoclonal and AGE-RNase (ribonuclease) for polyclonal antibody, for 1 hr at 37°C at a concentration of 0.5–1 mg/ml. The preabsorbed complexes were removed by centrifugation at top speed for 30 min. The supernatant was applied to the known AGE-positive spinal cord and motor cortex sections and the procedure for immunohistochemistry carried out as usual. Background immunostaining was assessed by omission of the primary antibody as another test for a negative control.

Results

SOD1, ubiquitin, and neurofilament immunoreactivities were co-localized with axonal spheroids and conglomerates as previously reported, along with the metabolites and enzymes related to nitric oxide/cGMP cascades (11,12). Immunoreactivities for all the AGEs tested were unequivocally intense and selective in both axonal spheroids (Fig. 1a, c) and conglomerates (Fig. 1b, d), whereas staining in the perikarya and the neuropil were negligible or equal to that of the background. AGE immunoreactivities in glial cells were also negligible, as were ubiquitin-immunopositive hyaline inclusions. The distribution patterns and immunoreactivities with antibodies against the two different AGEs were similar (Fig. 1a–d), although the monoclonal antibody against the AGE (cypentidone/piperidine-enolone) was most sensitive and selectively immunoreactive for neurofilament conglomerates with little background staining at a 1:24,000 dilution. Negative controls for both polyclonal and monoclonal (rabbit IgG and mouse IgG) immunostaining are shown in Figure 1e and 1f, respectively, after pre-absorption with corresponding antigen. Whereas the immunoreactivities for pyrrolidine were equally intense and selective for neurofilament conglomerates and axonal spheroids (Fig. 2a, b), those for pentosidine were not as selective as pyrrolidine (Fig. 2d). Ubiquitin immunoreactivity was most intense in hyaline inclusions (Fig. 2c, arrowhead) and axonal spheroids were modestly immunopositive as well, but the neuronal soma was immunonegative. No AGE immunoreactivity was detected in hyaline or Bunina inclusions. Co-localization of immunoreactivities of AGE with those of neurofilament subunits, especially of the nonphosphory-

lated NF-np (Fig. 2e) and NF-L-np (Fig. 2f), was unequivocal. No qualitative difference was noted in the immunoreactivities of axonal spheroids and neurofilament conglomerates between familial and sporadic ALS cases. Although neurofilament conglomerates in Betz cells were far more frequently noted in the familial ALS cases, its significance could not be construed because of the small number of cases studied. No neuronal inclusions were encountered in motoneurons of the control case. The rare diffuse intraneuronal neurofilament deposits of aging were infrequently encountered in small cortical pyramidal neurons, but they appeared distinctly different from the conglomerates in ALS patients.

Discussion

The linkage of the SOD1 gene mutation to chromosome 21q22 in a small fraction of patients with familial ALS suggests that aberrant oxidation or free radical production may be involved in the pathogenesis of this disease (8). Oxidative modification of neurofilament protein may also play a role, according to the results of studies of transgenic mouse lines that overexpress human neurofilament subunits, and to data obtained with human SOD1 mutants in which neurofilament accumulation was observed, as was neuronal cytoskeletalopathy secondary to SOD1-gained toxicity (21,36–43). In a case of ALS with SOD1-induced toxicity, concurrent neurofilament disruption and accumulation were postulated to be a general mechanism for the pathogenesis of familial ALS (44).

Neurofilaments consist of long-lived cytoskeletal proteins with a slow turnover rate, and both early and late (Maillard) AGEs form in neurofilaments obtained from patients with diabetes mellitus and diabetic polyneuropathy (45–48), normal aging (32,49), and Alzheimer's disease (35,50). Although the particular type of glycation damage affecting neurofilaments has not been well documented, the vulnerability of neurofilaments to modification by the AGEs pyrrolidine or pentosidine has been suggested as a likely mechanism for producing irreversible, protein-protein cross-linking (47,48). Neurofilament protein subunits of both 200 kD (NF-H) and 160 kD (NF-M) have multiple repeat sequences of KSP (Lys-Ser-Pro) at their carboxyl termini, and these subunits would be potentially susceptible to glycation reactions that affect lysine residues. It is noteworthy that both phosphorylation and

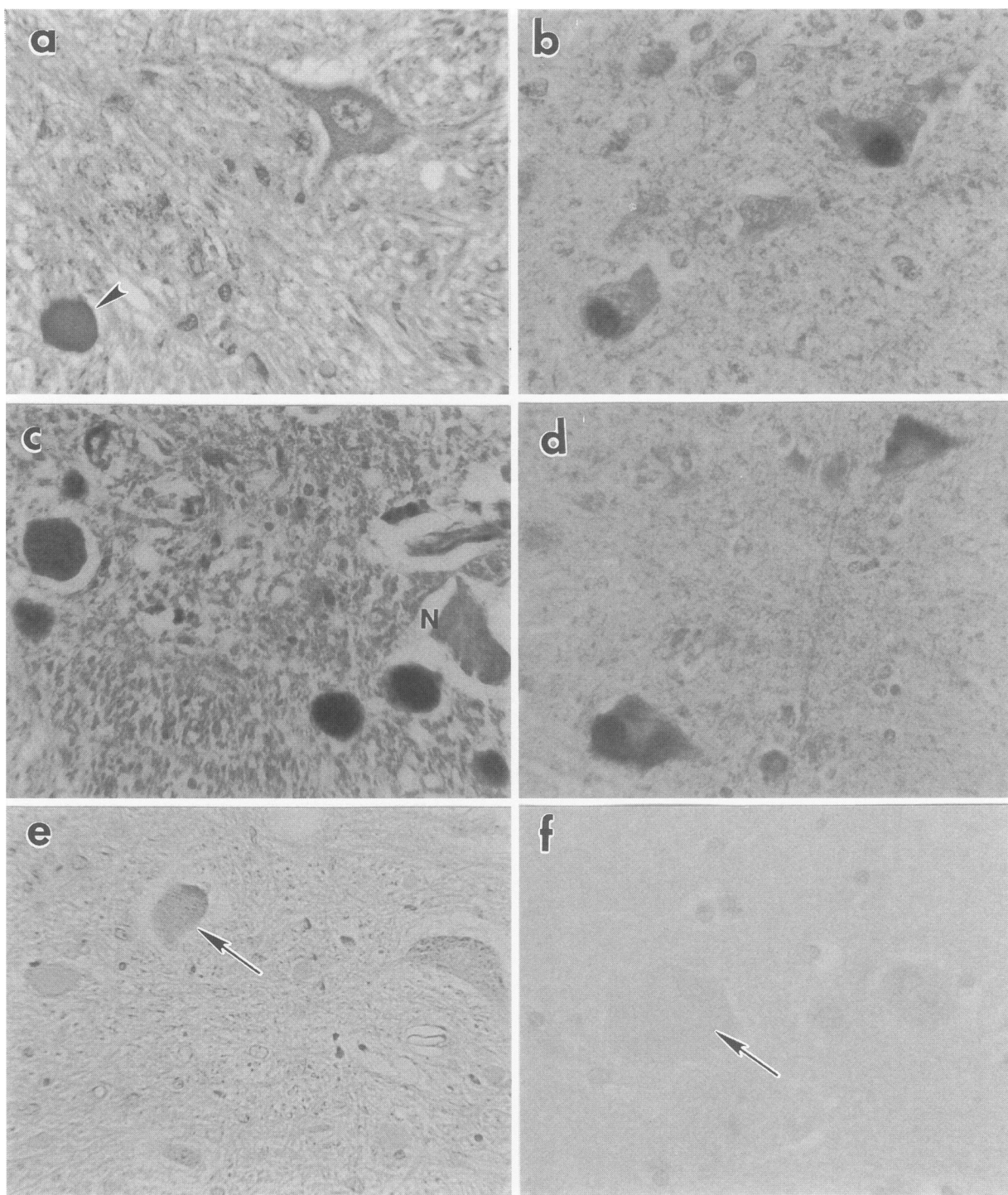


Fig. 1. Immunoreactivities for all AGEs tested in this study. (a) Anterior horn of the lumbar spinal cord of a case of sporadic ALS immunostained with polyclonal antibody against the AGE arg-lys imidazole. The arrowhead shows an immunoreactive axonal spheroid. Light counterstaining is with hematoxylin ($\times 320$). (b) Two pyramidal neurons in the motor cortex from a case of familial ALS showing spheroidal conglomerates immunostained with polyclonal antibody against arg-lys imidazole ($\times 300$). (c) Axonal spheroids intensely immunostained with a monoclonal antibody directed against the AGE cy-

pentodine/piperidine-enolone. N, neighboring spinal motor neuron ($\times 320$). (d) Two Betz cells containing conglomerates, immunostained with the anti-cy-pentodine/piperidine-enolone mAb ($\times 300$). (e) Faint AGE immunoreactivity in axonal spheroid (arrow) after preabsorption of polyclonal antibody with antigen protein (AGE-RNase) ($\times 300$). (f) Absence of AGE immunoreactivity in neurofilament conglomerate in a Betz cell (arrow) after preabsorption of monoclonal antibody with the antigen protein (AGE-BSA). ($\times 300$)

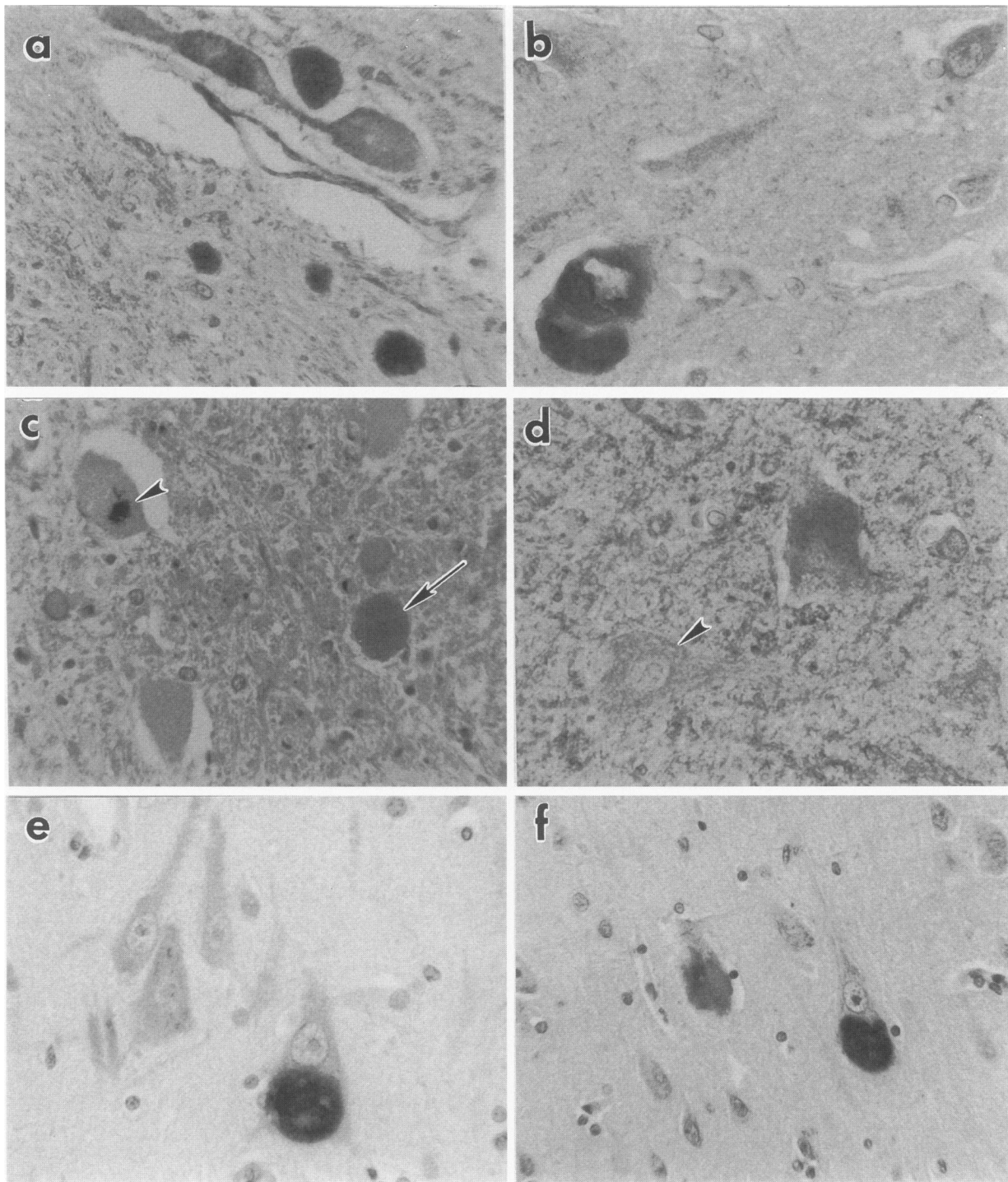


Fig. 2. Immunoreactivities for pyrraline, ubiquitin, pentosidine, and neurofilament subunits. (a) Anterior spinal horn showing scattered axonal spheroids intensely immunostained with anti-pyrraline mAb ($\times 320$). (b) A markedly swollen and deformed Betz cell with conglomerate strongly immunostained with the anti-pyrraline mAb and negatively immunostained central vacuoles ($\times 400$). (c) Intensely immunoreactive hyaline inclusion for ubiquitin (arrowhead) in neuron soma which is largely immunonegative and moderately immunopo-

sitive axonal spheroids (arrow) ($\times 300$). (d) Betz cell with conglomerate immunostained with an anti-pentosidine pAb. Note a Betz cell without conglomerate (arrowhead) free of immunoreactivity, serving as internal control ($\times 400$). (e) A round conglomerate intensely immunostained for nonphosphorylated neurofilament (NF-np) with a few vacuoles in Betz cells of which perikaryon is moderately immunopositive ($\times 400$). (f) Two Betz cells showing strongly immunopositive conglomerates for nonphosphorylated neurofilament light-subunit (NF-L-np) ($\times 300$).

enzymatic glycosylation of serine and threonine residues within the head domain of neurofilament subunits are considered essential for the normal assembly of neurofilaments, and these processes may be disrupted by AGE formation (51–55). AGE-modified proteins also can be degraded to yield reactive, low-molecular-weight AGE peptides that may further contribute to neurofilament damage and conglomeration, which is similar to what has been described for the reaction of AGE peptides with collagen or circulating low-density lipoprotein in vivo (30,56,57).

The present study provides indirect evidence that AGE formation may precede oxidative damage to neurofilament protein causing conglomeration and cross-linking (53,54). Indeed, the intense expression of SOD1 in neurofilament conglomerates and inclusions suggests that local production of superoxide may occur as a result of the glycation of neurofilament proteins (11,12,17). The production of superoxide and other radicals has been described in vitro in model glycation systems (58). Nitric oxide generation in motor neurons (which normally do not have the active enzyme for synthesizing nitric oxide) can be induced only by injuries such as axotomy or avulsion (59–61). Nitric oxide is also the only known biological molecule that is sufficiently reactive and produced in high enough local concentrations to compete with SOD1 for superoxide (9). Peroxynitrite, the reaction product of superoxide and nitric oxide, a potent oxidant, may then further contribute to the post-translational damage of neurofilament proteins. Accordingly, both motor neuron injuries and glycation of neurofilament protein may trigger and perpetuate peroxynitrite toxicity and the irreversible formation of neurofilament conglomerates.

Three post-translational neurofilament protein modifications have thus far been implicated in ALS. They are nitration, hyperphosphorylation, and glycation, all of which have been associated with oxidative damage of neurofilament proteins (10,47,62). Moreover, at least three major cytoplasmic proteolytic systems, balanced by their inhibitors, appear to be involved in the degradation of damaged neurofilament proteins (ubiquitin/ATP-dependent protease, serpin/serine protease, and cystatin/cysteine protease) (2,12). The activities of these systems overlap one another and are functionally interrelated in vivo. By immunohistochemistry, up-regulation of protein-bound nitrotyrosine was recently

demonstrated in the neurofilament conglomerates and axonal spheroids of motor neurons in ALS by labeling with nitrotyrosine antibody (11,12,23,24). Because of the abundance of tyrosine residues, the NF-L subunit may also be vulnerable to nitration by peroxynitrite, which is formed by nitric oxide and superoxide (9,10). Although a recent report (25) indicates that nitrotyrosine formation predominantly affects the free amino acid tyrosine and not tyrosine contained in protein residues, nitrated and glycated peptides may form small soluble “second-generation AGEs” that can further propagate protein and tissue damage (25,50,56).

The formation of AGEs on neurofilament proteins by a direct chemical reaction involving substrates other than reducing sugars cannot be excluded, as pyrroline production or “pyrrolylation” by 2,5, hexanedione (63) or by β - β' -iminodipropionitrile (IDPN) (64) may be possible. AGE formation may also occur, at least in part, after conglomerates form, as these aggregates are long-lived in vivo and may accumulate a variety of secondary modifications over time (30). In the present study, the most consistent and intense signals for AGE immunoreactivity co-localized with axonal spheroids and conglomerates. Investigation of toxic, second-generation AGE-peptides and recently identified AGE-specific receptors, which are abundantly distributed in bovine spinal motor neurons, is also warranted in ALS (34,56,65,66). Structural elucidation of the precise neurofilament modifications present in axonal spheroids and conglomerates may prove to be important in understanding the morphogenesis of the neuronal inclusions that characterize ALS.

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