Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease, with marked degeneration of the upper and lower motor neurons of the brain and spinal cord. Although the molecular events that underlie motor neuron death are not well defined, oxidative stress clearly contribute to the neuronal cell death in sporadic ALS (sALS), as evidenced by the elevation of markers for oxidation of protein, lipid and DNA. Most prior studies suggest that the changes are confined to motor neuronal populations of spinal cord and motor cortex in sALS. However, the extent of oxidative injury in the CNS has not been precisely determined and the distribution of the cells undergoing oxida-
tive injury is still controversial. Recent data suggest that the pathological processes in sALS are not limited to neurons but also involve glia in a significant way. Alterations of mitochondria in peripheral lymphocytes, platelets, motor nerve endings and liver, as well as, systemic impairment of glutamate uptake. sALS have been described and could well be secondary to widespread stress. Moreover, further studies showing the degeneration of other systems extending far beyond the motor neurons, decreased global regional cerebral blood flow and white matter damages in sALS support the possibility of a more widespread damage of the central nervous system (CNS) in sALS, than it was previously considered.

In our previous study, it was demonstrated that PARP-IR (IR) was increased both in the white and in the gray matter in sALS spinal cord, predominantly in cells with astroglial morphology. The present study was designed to evaluate whether the PARP expression is widespread in different regions of the brain in ALS including motor cortex, parietal cortex and cerebellum. The purpose was to delineate the extent of PARP related cellular stress in brain, including the regions different from motor cortex and in different cell types from motor neurons.

Materials and Methods

1. Postmortem tissue of s-ALS and control patients

The brain specimens were obtained from autopsy tissue from eight sporadic ALS patients followed in our MDA/ALS Clinic at Baylor College of Medicine (n=4) and University Maryland Brain Bank (n=4). Postmortem samples of brain from age-matched individuals without neurological diseases (n=4), and from a patient with Alzheimer disease were used as controls. The clinical diagnosis was confirmed pathologically in all cases. There was no difference between the average time from death to autopsy in ALS and control groups (8.0±6.9 hrs and 9±5.7 hrs). Additionally, there was no significant difference between the age at death (ALS 57.4±11.4 years, controls 51.8±15.8 years). In the ALS group, the causes of death in all cases were respiratory failure (n=8). In the control group, the causes of death were traffic accident, acute drug poisoning, and sudden death of unknown cause (2 cases). The Alzheimer case died of pneumonia. At autopsy, halves of the removed brains were fixed with immersion in 10% buffered formaldehyde and were subjected to neuropathological examination. The other halves of the brains were immediately frozen on dry ice and stored at -80°C until it was used to assay for western blotting.

2. Western blot

Samples to be analyzed were taken from the frozen brain specimens by separating small pieces (approximately 0.5 g) from the motor cortex, parietal cortex and from the cerebellum without thawing the tissue. Samples were homogenized with Wheatman homogenizer on ice in 20 mM HEPES buffer (pH 7.6, containing 200 mM KCl, 2 mM EGTA, 2 mM EDTA, 10 mM sodium molybdate, 50 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 0.1% NP-40, 10% glycerol, 4 mM benzamidine, 15 μg/ml aprotinin, 50 μM leupeptin, 1 μM pepstatin, and 1 mM phenyl-methyl-sulfonyl fluoride). The homogenized samples were sonicated twice with Sonic 300 Dismembrator (Artek, Farmingdale, NY) for 20 seconds. Protein content was measured by the Bradford method (BioRad, Hercules, CA). PARP, PAR, and caspase 3 IR were determined by immunoblot analysis. PARP was detected using the purified mouse anti-human PARP monoclonal antibodies 7D3-6 and C2-10 (Pharmingen, San Diego, CA). PAR was assayed using anti-poly (ADP-ribose) rabbit polyclonal antibody SA-276 (BioMol, Plymouth Meeting, PA). Goat polyclonal antibody, CPP32 (R&D systems, Minneapolis, MN) and rabbit polyclonal antibody, CM1(IDUN Pharmaceuticals, La Jolla, CA) were used to detect caspase-3. CPP32 was used to demonstrate both pro-caspase-3 and activated-caspase-3, while CM1 detected only the activated form of caspase-3. Samples from ALS patients and controls were subjected either to 8% (for PARP and PAR), or to 4-15% (for caspase-3) gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes by electroblotting. The nitrocellulose membranes were blocked for 1-2 h at room temperature with 5% powdered
milk in phosphate buffer saline containing 0.05% Tween-20 (PBST), then individually probed for either PARP (1:1000 for 7D3-6 and C2-10), PAR (1:2000), or caspase-3 (1:1000-2000 for CPP32 and CM1). The membranes were processed using a horseradish peroxidase-conjugated anti-mouse, anti-rabbit, or anti-goat secondary antibody and their signals were detected using ECL chemiluminescence (Amersham Pharmacia, Piscataway, NJ). The reliability of sample loading and electroblotting in each experiment was evaluated by stripping the membranes (Reprobe, Geno Technology Inc., St Louis, MO) and reprobing the blots with an antibody to actin (A4700; Sigma, St Louis, MO). Densitometric analyses were conducted using Image-J software obtained from NIH image systems.

3. PARP immunocytochemistry

Representative sections of brains from two ALS patients, from two normal controls and from the Alzheimer’s disease patient were immunostained for PARP. Brain specimens were fixed in 10% neutral formaldehyde and routinely embedded in paraffin. From paraffin blocks, sections of 5 μm thickness were cut, deparaffinized in xylene, rehydrated in descending series of ethanol, rinsed in 10 mM phosphate buffered saline (PBS), then were blocked for endogeneous peroxidase activity (0.3% H₂O₂ in methanol, 30 min). Sections were rinsed in PBS, then were placed in Serotec unmasking fluid (Serotec Inc. Raleigh NC) for antigen retrieval in a microwave oven (2×5 min at 600 W). After antigen retrieval the sections were cooled to room temperature and washed in PBS followed by a 30 min incubation with 5% normal horse serum (Vector Laboratories, Burlingame, CA) in PBS to block the nonspecific IgG binding sites. Mouse monoclonal anti-human PARP antibody(Serotec US, Raleigh, NC), diluted to 1/100 with PBS containing 2% horse serum was then applied at 40°C, overnight. After rinsing in PBS, sections were incubated with biotin-labeled horse-anti-mouse IgG (1/200 dilution in PBS, containing 2% normal horse serum; Vector Laboratories, Burlingame, CA) for 30 min at room temperature, rinsed in PBS and further incubated with a biotin-avidin complex containing also peroxidase for 30 min (Vector Elite kit; Vector Laboratories, Burlingame, CA). After a thorough wash in PBS the immunoreaction was visualized by 15 minute exposition to ImmunoPure metal enhanced DAB substrate kit (Pierce, Rockford, IL). The peroxidase reaction was terminated by extensive washing in distilled water. Finally, sections were dehydrated in graded series of ethanol, cleared in xylene and were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). As method control the primary antibody(mouse monoclonal anti-human PARP) was omitted from the reaction or was replaced with 1:100 dilution of normal mouse serum.

Immunostained sections were examined in a Zeiss Axioskop microscope (Karl Zeiss, Oberkochen FRG) equipped with a DXC-970-MD CCD camera (Sony Corp., Japan) and a digital image analysis system (Optimas 6.2; Optimas Corp., Bothell, WA).

All pyramidal neurons were counted using 125x magnification in 5 randomly selected areas of the III and V layers of the motor cortex of the ALS and control patients. Cells having positive (dark brown, black) nuclear staining for PARP were also counted in the same fields. In the parietal cortex, the large neurons were counted regardless of their shape. Then the neurons immunostained for PARP were counted in the same areas. In the sections of the cerebellum the Purkinje cells were counted in 5 randomly selected areas; then the immunostained cells were counted as well. The data were pulled to individual patients and were expressed in percent of immunostained neurons compared to the total number of counted cells. The density of subcortical immunopositive cells was arbitrarily chosen 100% in a disease control (Alzheimer’s disease) brain. All of the measured densities in the subcortical white matter of other brains were compared to this value in percent. Similarly, the measured density of immunostained glial cells in the cerebellar white matter of the Alzheimer patient was arbitrarily determined as 100%. The densities of the immunostained glial cells in the cerebellar white matter of the ALS patients and normal controls were expressed as a comparison to this value in
percent.

4. Statistical analysis

All values in the figures and text were expressed as mean±standard deviation. Normalized PARP protein levels characterizing ALS and control groups were analyzed with unpaired Student’s t-test, assuming unequal variances. Differences were considered statistically significant with p values of less than 0.05.

Results

In ALS specimens, neuronal losses with mild to moderate gliosis were observed in spinal cord and motor cortex, but no remarkable pathological changes were noted in other brain regions. No pathological changes in the CNS were present in the normal controls. In the disease control brain the pathologic criteria were compatible with Alzheimer’s disease.

1. PARP expression in different brain regions of patients with sALS

To determine the levels of PARP immunoreactivity in sporadic ALS, the amount of PARP protein was examined using immunoblot analysis of samples from different brain regions from ALS patients and age-matched controls. PARP-IR was increased in the sALS motor cortex, parietal cortex, and cerebellum compared to controls, as exemplified by reproducible immunoblots of selected samples (Fig.1 A, B, C). Quantitative comparison of the pooled data of the ALS (n=8) and normal controls (n=4) revealed that PARP-IR in motor cortex was approximately 2.5 times higher in ALS than in controls (p=0.006)(Fig. 1 D, left). In addition, the level of PARP in the parietal cortex and cerebellum was about 1.6 times higher than that of normal controls (p=0.043, p=0.035, respectively) (Fig.1 D, middle and right). The levels of PARP in the motor cortex of ALS patients were significantly higher than in the parietal cortex (p=0.03) or in the cerebellum (p=0.04). We could not demonstrate any PARP cleavage products (Fig. 1 A, B, C).

When compared to Alzheimer’s brain, the PARP-IR of motor and parietal cortex of ALS brain was more prominent. The PARP-IR of Alzheimer’s brain was higher than normal control, but less than that of ALS (Fig. 2).

2. Immunohistochemical staining for PARP

The density of the pyramidal cells was decreased, especially the gigantocellular pyramidal cells were lost in the V layer in the motor cortex of the examined ALS brains. Most of the remaining pyramidal cells expressed a strong dark brown or black nuclear staining leaving the nucleolus unstained (Fig. 3 A, B). The normal cell density was preserved in the motor cortex of the control cases including Alzheimer’s disease. However, the proportion of the cells immunostained for PARP was significantly lower in normal control (Fig. 3 E, F) and in Alzheimer (Fig. 3 C, D) brains, than in the ALS cases (Table 1, Mann-Whitney U-test: p<0.05). The intensity of the staining was also lower in the control brains.

The subcortical cells (glia and infiltrating macrophages) expressed high intensity and density of nuclear staining for PARP in both ALS cases. Determining the density as 100% in an Alzheimer patient’s brain, one of the ALS cases proved to be increased to 555%, while the other one increased to 488% (Fig.7). The staining pattern is illustrated (Fig. 4 A, B). The proportions of immunostained neurons in sections from the parietal cortex of ALS patients (Fig. 5

Table 1. Percentage of PARP immunoreactive neurons in different regions of ALS and control brains

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<th>Motor cortex</th>
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PARP; poly (ADP-ribose) Polymerase, ALS; Amyotrophic lateral sclerosis, ALZ; Alzheimer’s disease, NORM; normal control
Figure 1. PARP immunoreactivity in different regions of brain of sALS and normal controls. A, B, C: representative ECL photograph of immunoblot demonstrating increased PARP-IR in homogenates of various regions of brain (motor cortex, parietal cortex, cerebellum) of sporadic ALS and normal controls. Each well is loaded with 100 μg of total protein and blots are probed with anti-PARP monoclonal antibody (7D3-6). The same membranes is also probed with anti-actin antibody (after stripping) as an internal control. The positions of standard molecular weight markers in kilodaltons are indicated on the left of the blot. D: Quantitative data expressing PARP-IR levels, normalized to actin immunostaining, in the ALS (n=8) and control (n=4) groups. Each patient’s data (from three independent experiments) is averaged to obtain the means, +/- standard deviations (error bars), in the two groups. Data of sALS patients are expressed as percentage of simultaneously assayed normal control’s value. PARP expression in the motor cortex, parietal cortex and cerebellum of sporadic ALS patients is significantly higher than in controls (* p<0.01, ** p<0.05).
A) were also higher than in the controls (Fig. 5 C) (Table 1). The density and intensity of PARP expression in the subcortical glial cells were similarly high as which were measured in the frontal motor region (increased to 510% and to 477%) (Fig. 5 B). The proportion and intensity of PARP immunostaining of Purkinje cells in the cerebellum of the two ALS patients did not differ from that was found in Alzheimer’s disease control cerebellar tissue (Table 1). The staining pattern is illustrated (Fig. 6 A, C). However, the cells of the granular layer showed stronger immunostaining in ALS brain (Fig. 6 A), than in the cerebellum of Alzheimer disease patients (Fig. 6 C) or normal controls (Fig. 6 E). The densities of the PARP immunopositive glial cells in the white matter were also a little higher (130% and 125% in the ALS cases considering the value 100% in the cerebellum of Alzheimer case). The staining pattern is illustrated in Fig. 6 B and D. The Purkinje cells and also the subcortical glial cells stained a little less intensely in normal controls than in Alzheimer’s disease cerebellum (Fig. 6 E, F, and Fig. 7).

Summarizing the data, the density of pyramidal cells in the motor cortex of ALS patients is decreased. However, the cells remained intact expressed significantly higher density and intensity of immunostaining for PARP than the pyramidal cells in the same regions of control brains. The increased expression of PARP immunoreactivity was not confined to the motor cortex in ALS patients. Similar high expression was noted also in neurons of the parietal cortex. Furthermore, the expression of PARP was also increased in glial cells and in infiltrating macrophages in the subcortical white matter of both the frontal and the parietal lobes. The intensity and density of PARP immunostaining

Figure 2. Comparison of PARP expression in motor cortex and parietal cortex of ALS, normal control, and Alzheimer’s disease
Figure 4. PARP-IR in subcortical regions of motor cortex of ALS and Alzheimer’s disease.
A. The dark nuclei of glial cells and infiltrating macrophages express high intensity of immunostaining for PARP in the subcortical white matter of the motor region in the brain of an ALS patient. B. The nuclei of the glial cells in the subcortical white matter of the motor region of the brain of an Alzheimer patient show minimal intensity of immunostaining for PARP. A and B: peroxidase reaction, x120.

Figure 5. PARP-IR in parietal cortex of ALS and control. A. Strong immunoreactivity for PARP as dark staining in the nuclei of some of the neurons in the parietal cortex of an ALS patient. B. The nuclei of the subcortical glial cells in the same region also express strong immunoreactivity for PARP. C. There is no immunostaining for PARP in neurons of this region of the parietal cortex of a normal control brain. D. The subcortical glial cells in the parietal cortex of normal control brain show very slight immunostaining for PARP. A, B, C and D peroxidase reaction. A; x120, B; x240, C; x20, D; x240.
Figure 6. PARP-IR in cerebellum of ALS, Alzheimer’s disease and normal control. A. The nuclei of the Purkinje cells in the cerebellum of an ALS patient are strongly immunostained for PARP. Temperate immunostaining is noted in the cells of the granular layer. B. The nuclei of glial cells in the cerebellar white matter of an ALS patient express moderate staining intensity for PARP. C. Strong immunostaining of the nuclei of Purkinje cells for PARP in the cerebellum of an Alzheimer patient. D. The nuclei of glial cells in the white matter of the cerebellum of an Alzheimer disease patient are also moderately immunostained for PARP. E. The nuclei of the Purkinje cells are slightly immunostained for PARP in a normal control cerebellum. F. The immunostaining for PARP is lighter in the glial cells of the white matter of a normal control cerebellum. A, B, C, D, E and D; peroxidase reaction, x120. PARP: poly (ADP-ribose) polymerase
in the cerebellum of ALS patients seemed to be little higher than which were noted in the cerebellum of Alzheimer’s disease and normal controls.

**Discussion**

In our previous study, we demonstrated that the level of PARP IR in spinal cord from sporadic ALS was increased. The present study shows that PARP expression is also increased in motor cortex, parietal cortex and cerebellum in sporadic ALS patients compared to age-matched controls. Thus increased PARP expression in sporadic ALS is not limited to the motor system, it is rather widespread throughout the central nervous system.

To see the cellular distribution of increased PARP, immunohistochemical examination was carried out in sections of selected brain samples. Higher proportion of the pyramidal cells remaining morphologically intact in the motor cortex of ALS patients expressed increased PARP-IR in the nucleus compared to the pyramidal cells in Alzheimer disease brain or in brains of individuals not having CNS disease. It reflects the enhanced activation of DNA repair systems and increased consumption of NAD+ by the affected cells as a response for single or double-strand breaks in DNA. The present findings that this DNA repair enzyme is overexpressed in upper motor neurons in sporadic ALS is in concert with previous reports of oxidative damage to DNA and other macromolecules in this disease. The increased amount of the PARP content in the cells may correspond an enhanced activation of the enzyme. It results in an excessive consumption of the substrate (NAD+) of the enzyme. The depletion of energy of the cells can lead to apoptosis and necrosis. Consequently, the activation of PARP in upper motor neurons contributes to the development of cell degeneration. So far, the abnormal activation of PARP was shown to be involved in the development of cell damage in MPTP induced Parkinson disease, Alzheimer disease, ischemic brain damage and in CNS traumas. Thus, it can contribute to the degeneration of upper motor neurons at the level of individual cells. The up-regulation may be temporary and preceded or followed by a down-regulation of PARP in an earlier or later stage of the stress reaction of the cells as it was observed in spinal motor neurons. However, neurons in the parietal cortex of ALS brains express increased PARP in almost similarly high proportion as the motor cortex. They are not known to degenerate in ALS, so the enhanced expression of PARP in them can be the consequence of a widespread cellular stress. Nevertheless these cells survive, while motor neurons lacking some self protecting mechanisms (e.g.: having low level of parvalbumin) are gradually destroyed.

Even if the density of neurons is decreased in the motor cortex, this area showed the highest level of PARP in immunoblots. It is explained by three different factors. The proportion of the decreased number of PARP positive neurons is higher, than in control neurons. The region is heavily infiltrated by macrophages, which normally highly express PARP in their nuclei as we observed it in the ventral roots of ALS spinal cords. Finally astrogliosis is a common phenomenon in the region of degeneration. Increased number of hypertrophic astrocytes also express increased amount of PARP. However, astrocytes contained high amount of PARP in the parietal cortex too. The fact that glia in ALS spinal cord and brain have increased PARP-IR suggests that cells other than only neurons may be subjected to pathologic stressors in ALS, and in turn, such altered glia may contribute to the pathogenesis of ALS. The Purkinje cells in the cerebellum seem to be protected from PARP increase (containing high amount of parvalbumine), while the white matter glial cells and the...
cells in the granular layer contain more PARP than in control brains. However, the magnitude of increase may not be enough for inducing neuronal damage clinically and neuropathologically.

The widespread cellular stress in the CNS of ALS patients can be mediated by calcium channel antibodies, leading to increased intracellular calcium. PARP can also be activated by inositol 1, 4, 5, -triphosphate-Ca mobilization without DNA damage. Ca promotes activation of PARP and Ca is an activator factor for cell killing.

Microgliosis may be the other source of generalized stress. During activation, the external membrane of the microglia cells and macrophages releases a large amount of free radicals, damaging the cell membrane of the neighboring neurons and the astroglial glutamate transporter. The microglia can enhance the neuronal injury by releasing cytokines, and by secretion of glutamate. All of these factors lead to raised intracellular calcium, increased level of free radicals and subsequent DNA damage and PARP activation. As the harmful factors are soluble, they can reach the structures far from their site of release in the motor system. The injury of further structures depends on their distance from the release sites (which may influence the concentration of these harmful agents) and also from their capability of self protection, primarily from calcium stress.

Increased activity of PARP can be an add-on injury for the cells. The excessive consumption of NAD+ depletes the internal energy source of the cells, enhances the ATP deficit. The resulting energy failure jeopardizes the function of ATP-requiring ionic pumps which maintain the electrochemical gradients across neuronal plasma membranes. Insufficient clearance of calcium induces a further rise in intracellular calcium, which initiates a variety of enzymatic processes leading to cell death.

As we stated earlier, spinal motor neurons express lower level of PARP in ALS tissue than in control ones. Thus they behave exactly opposite to pyramidal neurons. The spinal motor neurons in sporadic ALS at the beginning of the degenerative process might be able to give priority to other highly ATP-dependent processes, such as ubiquitination and proteosomal removal of aggregated proteins, decreasing intracellular calcium, enhancing altered axoplasmic transport and preserving neurotransmitter release from an increased number of sprouted axon terminals in ALS. Especially this later function is specific for lower motor neurons and not needed for pyramidal cells. Further studies should be performed to define the exact role of enhanced PARP expression in astrocytes.

Although we did not demonstrate the precise mechanisms of increased PARP in neuron and glial cells, the findings that PARP-IR was present in both cortical neuronal and subcortical glial cells and which was not limited only to the vulnerable motor cortex suggest that widespread cellular stress on neuronal and glial cells is present in the brain of sporadic ALS patients.

REFERENCES

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