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c-Jun, JNK/SAPK Kinases and Transcription Factor NF-κB Are Selectively Activated in Astrocytes, but not Motor Neurons, in Amyotrophic Lateral Sclerosis

ANTONIO MIGHELI, MD, PHD, ROBERTO PIVA, PHD, CRISTIANA ATZORI, PHD, DIRK TROOST, MD, AND DAVIDE SCHIFFER, MD

Abstract. There is increasing evidence that oxidative damage plays a major role in amyotrophic lateral sclerosis (ALS), but how it contributes to motor neuron degeneration and astrocytic gliosis, two pathologic hallmarks of the disease, is unknown. A few studies have suggested that ALS motor neurons die via apoptosis and show upregulation of c-*jun*, an immediate early gene that is necessary for neuronal apoptosis. In order to elucidate the mechanisms of cell damage induced by oxidant stress, we have studied in ALS and control spinal cord the immunohistochemical expression of c-Jun, of JNK/SAPK, a kinase that activates c-Jun following various types of stress, and of NF-κB, a transcription factor that is induced by oxidant stress and has prominent neuroprotective functions. An in situ end-labeling assay was performed for detecting apoptotic cells. We show that (a) the JNK/SAPK-c-Jun pathway is dramatically overexpressed in ALS spinal cord; (b) the strongest activation occurs in astrocytes, while motor neurons show unusually low expression of the pathway; (c) increased JNK/SAPK expression in glial cells is accompanied by NF-κB activation, indicating the presence of a protective response to oxidant stress, which is deficient in motor neurons; (d) activation of JNK/SAPK, c-Jun and NF-κB is unrelated to apoptotic cell death. These results support the view that astrocytes are directly involved in the pathologic process of ALS, and might explain the selective vulnerability of motor neurons by their relative lack of antioxidant defenses.

Key Words: Amyotrophic lateral sclerosis; Apoptosis; c-Jun; JNK/SAPK; NF-κB; Oxidant stress.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a human neurodegenerative disease characterized by progressive impairment of the motor system. Although both upper and lower motor neurons are clinically affected, pathologic changes prevail in the latter (1). Spinal cord, medulla oblongata and, to a lesser extent, the motor cortex show atrophy and loss of motor neurons and characteristic ubiquitinated filamentous inclusions in some of the remaining neurons (2). In addition, a prominent astrogliosis has been described in the motor cortex and spinal cord (3, 4). Both sporadic and familial forms are known; in a subset of the latter, missense mutations in the gene encoding the cytosolic enzyme Cu, Zn superoxide dismutase (SOD1) have been found (5), and studies on transgenic mice expressing the mutated enzyme suggest a correlation between enhanced intracellular oxidant stress and motor neuron degeneration (6).

The pathogenesis of most ALS cases is unknown; several factors, such as an impairment of anti-oxidant defenses, a primary cytoskeletal pathology, and glutamate-related excitotoxicity, have been proposed as possible culprits (7, 8). Because of the convergence of causal factors on the oxidant stress pathway, this is currently favored as a major determinant of motor neuron degeneration (7–9), although it has not been determined whether oxidative damage is an early- or end-stage phenomenon. How ALS motor neurons become dysfunctional and eventually die is also unclear. Besides atrophy and cytoskeletal abnormalities, features reminiscent of apoptosis, i.e. a type of death characterized by active transcription of "death genes," have been reported in ALS (10, 11), but not confirmed in other studies (12). As a matter of fact, while cellular RNA content is reduced in ALS motor neurons (13), some apoptosis-related genes were reportedly upregulated in ALS tissue (14, 15). In particular, the immediate early gene c-*jun* was found to be overexpressed in postmortem spinal cord of ALS patients (15). This observation is intriguing, since c-*jun* appears as a pivotal element in the decision of neurons to die via apoptosis (16, 17).

In order to elucidate the molecular cascade which leads to c-*jun* activation and to better understand its role in ALS, we have focused our attention on the JNK (c-Jun N-terminal kinase)/SAPK (stress activated protein kinase) intracellular signaling pathway (18, 19). The JNK/SAPK family of kinases comprises at least 3 isoforms, called JNK1 (SAPKγ), JNK2 (SAPKα), and JNK3 (SAPKβ) (18), all of which are capable of activating c-Jun following various types of stress (18, 19). In this report, the immunohistochemical expression of the 3 JNK/SAPK kinases was studied in the spinal cord of normal and ALS patients. The results were compared with those obtained with antibodies to NF-κB, a transcription factor which is strongly induced by oxidative damage and appears to

From the Department of Neuroscience, Laboratory of Neuropathology, University of Turin, Italy (AM, RP, CA, DS) and the Academic Medical Center, Graduate School Neurosciences Amsterdam, Department of Pathology, Division of Neuropathology, Amsterdam, The Netherlands (DT).

Correspondence to: Dr Antonio Migheli, Department of Neuroscience, University of Turin, via Cherasco 15, 10126 Turin, Italy.

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have prominent anti-apoptotic and neuroprotective functions (20, 21). Finally, we performed an in situ end-labeling (ISEL) technique for demonstrating the occurrence of DNA fragmentation possibly related to apoptotic cell death (12).

MATERIALS AND METHODS

Selection of Patients

Ten end-stage ALS and ten control cases were selected for the study. All cases were from two institutions—the Department of Neuroscience, Laboratory of Neuropathology, University of Turin, and the Department of Pathology (Neuropathology), University of Amsterdam—and had already been studied extensively (11, 22). Mean age at death was 61 years for ALS cases and 67 years for control cases. Duration of illness in ALS cases ranged between 18 and 54 months. Control patients were affected by either non-neurological diseases (n = 5) or by neurological diseases (brain infarcts or tumors) not involving the spinal cord (n = 5). All cases had been autopsied within a short time from death (4 to 10 hours). After fixation in formalin, blocks from cervical and lumbar spinal cord were dehydrated and embedded in paraffin. All reactions were performed on 5-μ-thick serial sections mounted on Superfrost™ slides.

Immunohistochemistry

After deparaffinization, rehydration, and blocking of endogenous peroxidase activity with 3% hydrogen peroxide, sections were stained with the following antibodies: (a) anti-SAPKα (Upstate Biotechnology) at 1/200 dilution; (b) anti-SAPKβ (Upstate Biotechnology) at 1/200 dilution; (c) anti-JNK1 (Santa Cruz Biotechnology) at 1/100 dilution; (d) anti-c-Jun/AP-1 (Oncogene Science) at 1/400 dilution; and (e) anti-NF-κB p65 subunit (Santa Cruz Biotechnology) at 1/100 dilution. All antibodies were rabbit, affinity-purified polyclonal antibodies raised against specific synthetic peptides. Specificity of the reaction was tested by incubating the antibodies with the respective immunizing peptides prior to immunostaining. Before staining with the antibody to c-Jun, sections were placed in 0.01 M citrate buffer, pH 6, and heated in a microwave oven (Whirlpool) at 750 W until boiling, then at 350 W for 15 min. Immune reactions were revealed with the streptABC technique, using cobalt chloride-intensified DAB (Boehringer) as substrate. Sections were counterstained with hematoxylin.

Quantitative Analysis

Immunostained nuclei were counted with an oil-immersion objective at 1000× magnification. On each half of spinal cord section, 3 areas (0.02 mm^2) were randomly chosen in each of the following locations: anterior horns, posterior horns, corticospinal tracts and posterior columns. The number of total cells/area and the number of immunostained nuclei/area were counted. For each parameter, the area values were added together and the percentage of labeled cells was determined in each case. A mean percentage was calculated for all ALS and control cases. Statistical analysis was performed with a Student's *t* test.

In Situ End-labeling (ISEL) Technique

Terminal deoxynucleotidyl transferase (TdT) was applied to tissue sections in order to incorporate labeled dUTP in the DNA breaks that may accompany both apoptotic and necrotic cell death (12). Deparaffinized sections were either left undigested or were first subjected to proteolytic treatment in order to increase the incorporation of labeled nucleotides. Tissue digestion was performed with proteinase K (Sigma) at concentrations ranging between 2.5 and 50 μg/ml in Tris-EDTA (TE) buffer for 15 minutes (min) at room temperature (RT). After blocking of endogenous peroxidase activity with 3% H_2O_2 for 10 min, sections were rinsed in TdT buffer (25 mM Tris HCl, 200 mM sodium cacodylate, 5 mM cobalt chloride) and incubated with the labeling mix (4-20 U TdT (Boehringer), 1 nmol fluorescein-11-dUTP (Boehringer) in 100 μl TdT buffer) for 120 min at 37°C. The reaction was terminated by rinsing in 300 mM sodium chloride-30 mM sodium citrate (2× SSC) for 10 min at RT. The sections were placed in Tris buffered saline (TBS) containing 0.3% Triton X-100 and 2% bovine serum albumin for 30 min at RT, then incubated with anti-fluorescein sheep antibody conjugated with peroxidase (Boehringer), diluted 1/500 in TBS-Triton, for 30 min at 37°C. After washing in TBS, the reaction was revealed with cobalt chloride-intensified DAB. Sections were counterstained with hematoxylin, dehydrated, and mounted in Permount. Control of the reaction was performed by omitting TdT from the labeling mix.

RESULTS

Effect of Control Reactions

Preincubation of the antibodies with the respective immunizing peptides resulted in complete abolishment of staining. Similarly, no staining was obtained with the ISEL reaction if TdT was omitted from the labeling mix.

Immunohistochemistry (Results Are Summarized in Table 1)

(a) *Normal spinal cord.* The expression of c-Jun and of all 3 JNK/SAPK kinases was limited to sparse cells, mostly located in the anterior horns and in the substantia gelatinosa of posterior horns (Fig. 1a). Large motor neurons showed a punctate cytoplasmic immunoreactivity, but no nuclear staining. Sparse glial cells displayed weak nuclear staining. Gliosis was absent in all cases; in one case (81-year-old subject with mild diffuse arteriolosclerosis), a small group of reactive astrocytes that showed strong nuclear staining for c-Jun and JNK/SAPK was present. NF-κB immunoreactivity was similar, with a relatively stronger labeling of neuronal perikarya (Fig. 1b).

(b) *ALS spinal cord.* Compared with control cases, the immunoreactivity for c-Jun and JNK/SAPK kinases was dramatically increased in selected areas such as laminae VII-IX of the anterior horns, the corticospinal tracts at the transition with the anterior horns, and the emergency of ventral roots from the anterior horns (Fig. 1c). Somewhat less conspicuous staining was also found in laminae I–III of the posterior horns. Other regions of the cord showed a very limited number of stained cells, with the least number occurring in the posterior columns. No significant differences in the amount and distribution of immunoreactivities were found between c-Jun and JNK/

TABLE 1
Percentages of JNK/SAPK, c-JUN and NF-κB Immunoreactive Cells in ALS and Control Spinal Cord

	JNK/SAPK		c-Jun		NF-κB	
	Controls	ALS	Controls	ALS	Controls	ALS
Anterior horns	2.5 (0–8.5)	43.2 (37–48.9)	1.2 (0–7.8)	36.8 (17.6–48.3)	4.9 (0–6.7)	48.4 (38.9–67.3)
Posterior horns	0.7 (0–2.9)	11.4 (5.2–15.3)	0.3 (0–1.3)	11.7 (9.3–19.3)	2.3 (0–8.5)	17.6 (10.5–27.1)
Corticospinal tracts	1.1 (0–2.4)	52.6 (41.8–64.2)	1.4 (0–2.9)	40.1 (23.4–58.1)	2.1 (0–3.4)	54.8 (43.4–62.5)
Posterior columns	1.8 (0–3.2)	6.7 (2.3–14.3)	1.1 (0–4.1)	6.2 (3.1–10.7)	1.3 (0–5.7)	7.4 (3.4–24.1)

Only cells showing nuclear immunoreactivity, an indicator of protein activation, were counted. Since no staining of neuronal nuclei was observed, all labeling belongs to glial cells. Values indicate the mean percentage, while the range of measurements is indicated in brackets. With all antibodies, significant differences were found between control and ALS patients for all tissue parameters ($p < 0.01$ for anterior and posterior horns and corticospinal tracts, $p < 0.05$ for posterior columns). No significant differences were found among the three assays.

SAPK, or between the 3 kinase isoforms, suggesting that c-Jun and SAPK α, β, and γ were all coexpressed in the same cells.

Examination of the cellular distribution of JNK/SAPK immunoreactivity revealed striking differences between neuronal and glial cells. Anterior horn motor neurons were either unstained or showed cytoplasmic punctate staining and absence of nuclear staining, a pattern indistinguishable from that seen in motor neurons of control cases (Fig. 1d, e). Scattered neurons of laminae I-III were similarly stained. The majority of labeling in the anterior horns occurred in astrocytes, both reactive and nonreactive (Fig. 1d, e). The former were identified by the presence of a well developed cytoplasm. Reactive astrocytes showed various types of immunoreactivity, i.e. strong nuclear staining only, less intense nuclear and cytoplasmic staining, weak cytoplasmic staining only, or no labeling at all. In the same areas, more frequent and stronger nuclear staining occurred in nonreactive astrocytes, i.e. astrocytes devoid of a clear-cut large cytoplasm. At the transition between the anterior horns and the corticospinal tracts, where a prominent gliosis has been described (4), both reactive and nonreactive immunostained astrocytes were frequently found (Fig. 1f). The rest of the white matter showed only scattered, immunolabeled cells with astrocytic features. Notably, all astrocytes of the posterior columns were unstained.

Immunostaining for c-Jun was identical to that of JNK/SAPK. Reactive and nonreactive astrocytes showed intense nuclear staining, while motor neurons were either unstained or showed punctate cytoplasmic staining (Fig. 2a, b). Increased c-Jun immunoreactivity has been shown to occur in the nuclei of cells undergoing apoptosis (16, 17). In ALS tissue, however, no JNK/SAPK- or c-Jun-immunoreactive cells showed clear-cut apoptotic changes such as nuclear pyknosis or fragmentation into apoptotic bodies.

The distribution of NF-κB immunoreactivity was identical to that of JNK/SAPK, with the majority of staining occurring in the astrocytes of anterior horns and at the transition between these and the corticospinal tracts. As with JNK/SAPK, enhanced NF-κB staining was seen in the nuclei of astrocytes with or without an evident cytoplasm, while the cytoplasm of reactive astrocytes was unconstantly labeled (Fig. 2c, d). In motor neurons, staining of perikarya was slightly more pronounced than with the anti-JNK/SAPK antibodies, but nuclei were unlabeled (Fig. 2d). As with antibodies to JNK/SAPK, astrocytes of the posterior columns were not stained for NF-κB (Fig.

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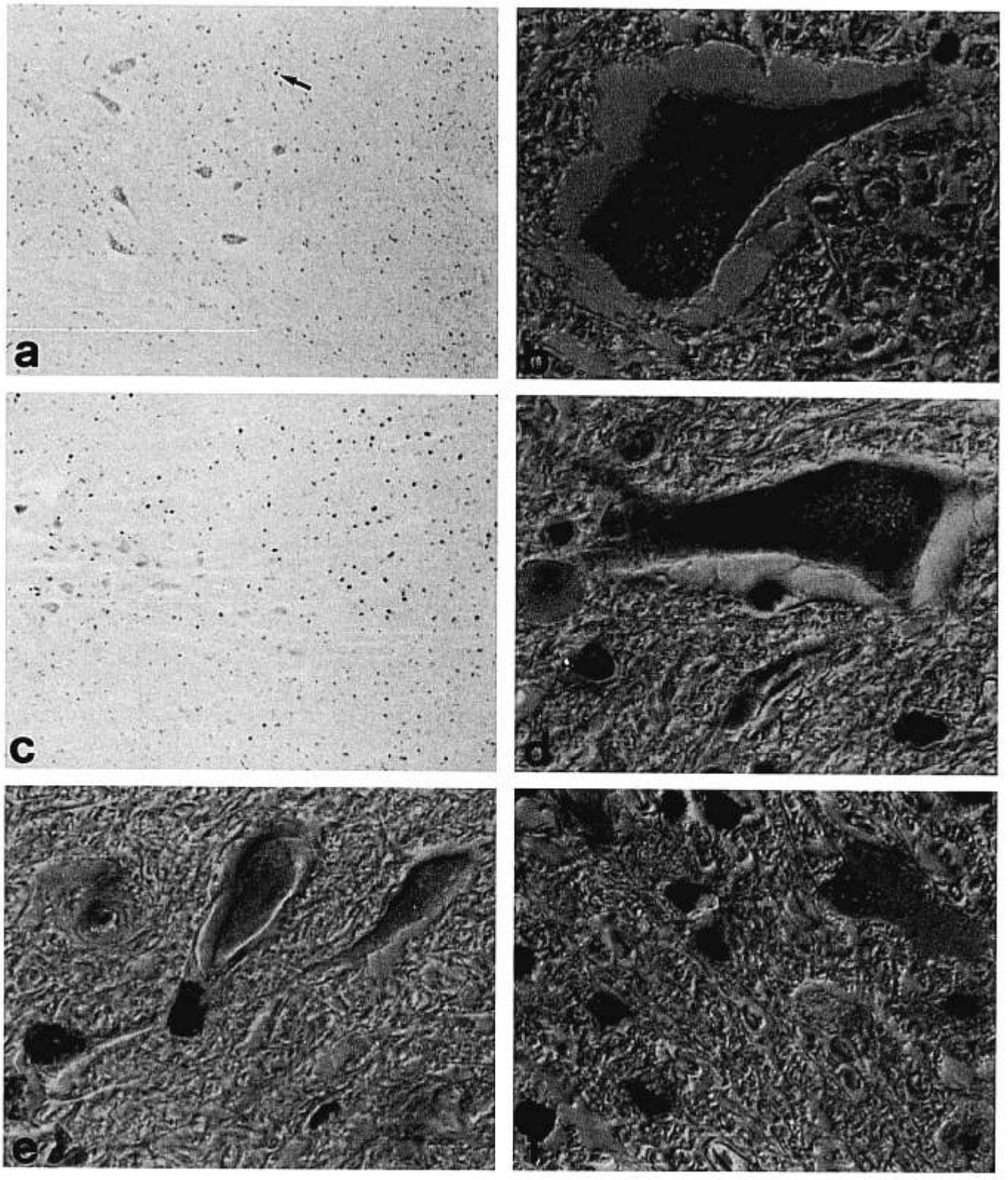
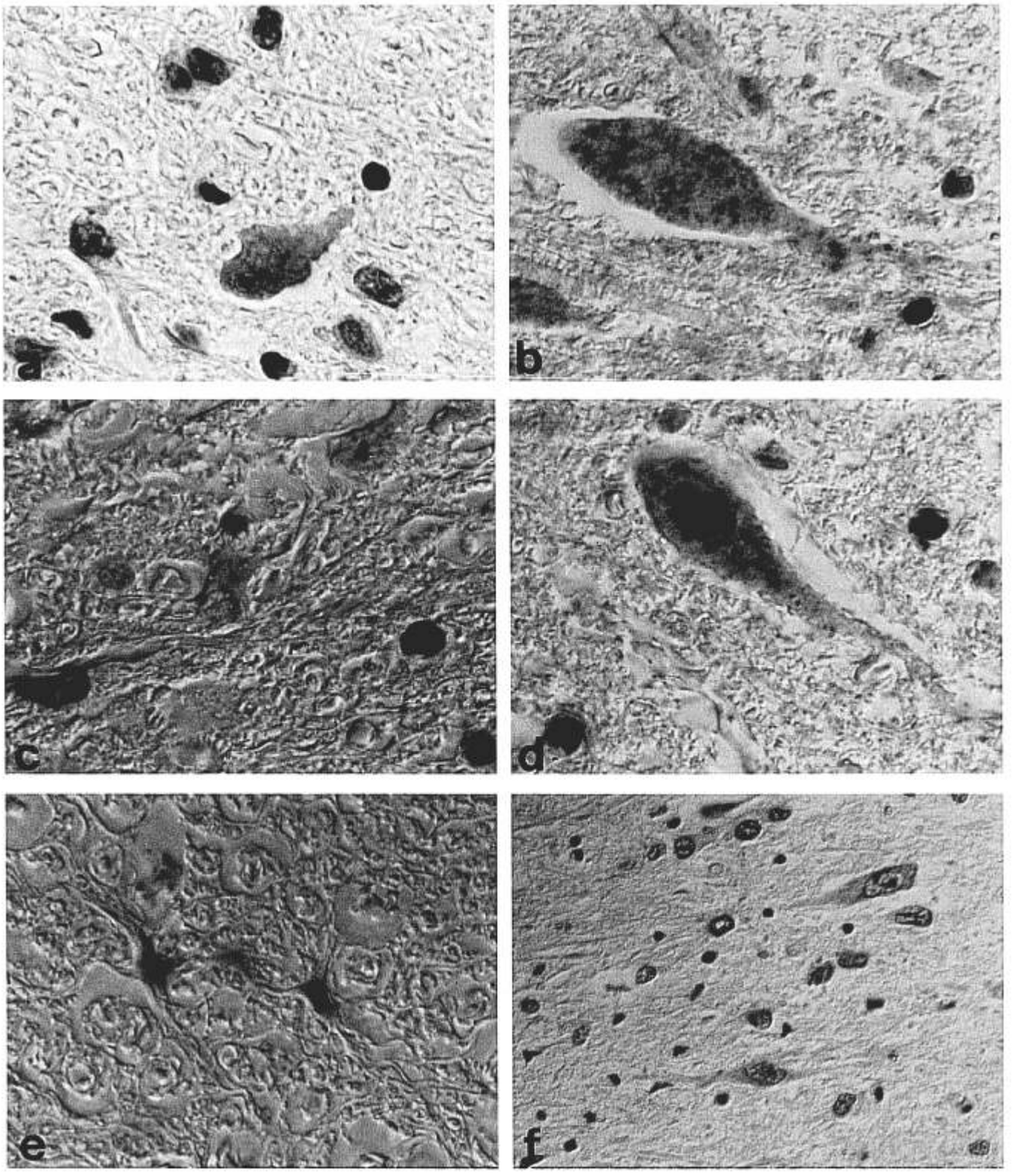
Fig. 1. (a) Expression of JNK/SAPK in normal spinal cord. Immunostaining is limited to very few glial nuclei (arrow) in the anterior horn. (b) Expression of NF-κB in a motor neuron from a control case. Punctate immunostaining is diffusely present in the cytoplasm; (c) Expression of JNK/SAPK in ALS spinal cord. Compared to (a), far more numerous immunostained nuclei are present in the severely atrophic anterior horn and in the corticospinal tract, at the transition with the anterior horn. White matter tracts below the anterior horn are virtually unstained. (d–f) Expression of JNK/SAPK in ALS spinal cord. In the anterior horns, motor neurons show diffuse punctate staining of the cytoplasm (d) indistinguishable from motor neurons of control cases (compare with b); more often, however, they are unstained (e). Several astrocytes, mostly the nonreactive type, show intense nuclear staining. (f) Staining of astrocytes at the transition with the corticospinal tracts. Nuclear staining is restricted to several nonreactive cells; a reactive astrocyte shows only scattered punctate cytoplasmic reactivity for JNK/SAPK. (a, c) ×100; (b, d–f) ×1,000.





2e). Again, no NF-κB–labeled cell showed an apoptotic morphology.

ISEL

In the absence of proteinase K pretreatment, or at low concentrations of TdT, no significant labeling of nuclei suggestive of DNA fragmentation was produced. At increasing concentrations of proteinase K and/or TdT, a progressively stronger labeling appeared in most cells throughout the section, without differences between ALS and control cases. The nuclear labeling was seen in neurons, glial and even endothelial cells (Fig. 2f). Furthermore, none of the ISEL-labeled cells displayed clear-cut apoptotic features, as has been well documented in several CNS diseases (12). Therefore, we considered the ISEL staining to be unrelated to either apoptosis or disease state, and to be most likely the result of postmortem DNA degradation (12).

DISCUSSION

In this paper we have shown that (a) the JNK/SAPK-c-Jun intracellular signaling pathway is dramatically overexpressed in the spinal cord of ALS cases; (b) the strongest activation occurs in astrocytes, while motor neurons show unusually low expression of the pathway; (c) increased JNK/SAPK expression in glial cells is accompanied by NF-κB activation, indicating the presence of a vigorous protective response to oxidant stress, which is deficient in motor neurons; (d) activation of JNK/SAPK, c-Jun, and NF-κB is unrelated to apoptotic cell death. These results support the view that astrocytes are directly involved in the pathologic process of ALS (23, 24), and might explain the selective vulnerability of motor neurons by their relative lack of antioxidant defenses. The key observations are discussed below.

JNK/SAPK kinases are a subgroup of the mitogen-activated protein kinases (MAPKs), which are important mediators of signal transduction from the cell surface to the nucleus, and also include the extracellular-signal-regulated kinases (ERKs) (18). Unlike ERKs, JNK/SAPKs are poorly induced in response to mitogens but are potently activated by proinflammatory cytokines and by various forms of environmental stress, such as UV, heat shock, hyperosmolarity, protein synthesis inhibitors, ischemia, DNA damage, and oxidant stress (18, 19, 25–27). JNK/SAPKs are present in both cytoplasm and nucleus in normal conditions, but translocate to the nucleus following stress stimuli (28). JNK/SAPKs are activated by a kinase cascade (29) that appears to require ceramide signaling (30). An association of the JNK/SAPK pathway with apoptosis has been shown in some cellular systems (29–31), but not confirmed in others (21, 32). Although JNK/SAPK kinases are capable of phosphorylating various cytoskeletal proteins such as MAP2, tau and NF-H (33, 34) in vitro, c-Jun appears to be a major target of their activity (35). Once phosphorylated in the nucleus, c-Jun becomes a component of the transcription factor complex AP-1, which regulates the transcription of genes involved in such diverse functions as cell growth, differentiation, regeneration and death (36, 37). In vitro studies have shown that JNK/SAPKs are activated in neurons during apoptosis induced by NGF withdrawal (31), but also during differentiation (38) and following glutamate stimulation (39). JNK/SAPKs are also expressed by glial cells under experimental paradigms relevant to astrogliosis (40). In the adult rat brain, constitutive expression of JNK/SAPKs has been found at the neuronal level (41).

The present data represent the first report on JNK/SAPK expression in human normal nervous tissue. In control cases, low but detectable levels of JNK/SAPKs and c-Jun were found in scattered neurons and astrocytes. Interestingly, neurons showed cytoplasmic-only staining, while astrocytes were mostly stained in their nuclei, suggesting nuclear translocation and activation of the pathway (28). Constitutive expression of the JNK/SAPK pathway is not unexpected, due to the many physiological processes which are under the control of c-Jun (37).

A comparison of JNK/SAPK expression in control and ALS spinal cord revealed in the latter a striking upregulation, which was consistently observed with all 3 JNK/SAPK antibodies as well as with the antibody to c-Jun. Upregulation mainly occurred in areas affected by pathologic changes, such as the anterior horns and at the transition between anterior horns and corticospinal tracts. The latter region corresponds to the entering points of the corticospinal fibers, and is the site of prominent gliosis (4) and neuritic dystrophy (42), as a consequence of corticospinal derangement. Furthermore, upregulation of the JNK/SAPK pathway was limited to glial cells, while neurons showed no increase in kinase and c-Jun expression

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Fig. 2. (a, b) Expression of c-Jun in ALS spinal cord. Reactive and nonreactive astrocytes show intense nuclear staining. Motor neurons are either unstained (a) or show punctate cytoplasmic staining (b). (c–e) Expression of NF-κB in ALS spinal cord. (c) Corticospinal tract: reactive astrocytes show either diffuse nuclear staining (left) or punctate cytoplasmic staining (center); two nonreactive astrocytes (bottom right) show strong nuclear staining. (d) Anterior horn: motor neurons show punctate cytoplasmic staining, while strong nuclear staining is seen in nonreactive astrocytes. (e) Astrocytes of the posterior columns are not immunoreactive for NF-κB. (f) In situ end-labeling in ALS spinal cord. Most cells in the section show diffuse nuclear labeling. None of the labeled cells has an apoptotic morphology. (a–e) ×1,000; (f) ×400.

compared with controls. In an earlier study, in which the Northern blot method was used, an increased expression of c-*jun* mRNA in ALS spinal cord was also found (15). However, analysis of the cellular expression by in situ hybridization revealed c-*jun* upregulation in motor neurons but not in astrocytes (15). Whether this discrepancy reflects posttranslational upregulation of c-Jun in astrocytes with absence of activation of the corresponding gene remains to be ascertained.

Induction of the JNK/SAPK pathway is an early cellular response to stress stimuli (35–37), and is reflected by the appearance of nuclear immunoreactivity (28). In ALS motor neurons, lack of nuclear staining for JNK/SAPK and c-Jun indicates that no induction of the pathway has occurred. On the contrary, the prominent nuclear staining seen in astrocytes is a strong evidence for activation of the pathway. Interestingly, the distribution of JNK/SAPK immunoreactivity among glial cells revealed that (a) not all astrocytes showing increased nuclear staining were reactive, i.e. had an enlarged cytoplasm with multiple processes; and (b) not all reactive astrocytes showed nuclear staining. In a likely scenario, activation of the JNK/SAPK pathway would precede the appearance of a phenotype with the characters of reactive gliosis, which need not involve further JNK/SAPK induction. In agreement with this interpretation, studies on primary glial cultures have suggested a role for JNK/SAPK activation in signaling for gliosis (40). It should be expected that activation of the same pathway will be present in other diseases in which gliosis is prominent. More work needs to be done in order to clarify this point.

What are the mechanism and significance of JNK/SAPK and c-Jun activation in ALS astrocytes? Oxidant stress appears to play a major role in ALS (6–9, 23, 24, 43). Accordingly, astrocytes of ALS spinal cord show enhanced immunostaining for manganese superoxide dismutase (SOD2), as a likely attempt at compensation for free radical injury (44). JNK/SAPK activation in ALS astrocytes might represent a protective response to oxidant stress, since the latter is a known inducer of the JNK/SAPK pathway (25–27). The present finding of an increased expression of NF-κB in the same glial population that overexpresses JNK/SAPK and c-Jun supports this interpretation. The transcription factor NF-κB is involved in cellular defense mechanisms activated by pathologic stimuli that induce oxidant stress (45–47). In addition, NF-κB appears to actively suppress signals for apoptotic cell death (21, 48–50). In astrocytes, NF-κB is induced by a variety of stimuli, such as cytokines, inflammation, excitotoxicity, and ischemia, all of which may promote oxidant stress (51–54). Since JNK/SAPK and NF-κB appear to be activated by the same spectrum of stimuli (21), these data strongly argue that the increased expression of JNK/SAPK and NF-κB in ALS astrocytes has a primary protective role from oxidant stress. This is also in agreement with the well-known neuroprotective role of astrocytes (55).

A further hypothesis originating from our findings is that the selective vulnerability of motor neurons in ALS might be linked to a defect in the antioxidant defense mechanisms represented by JNK/SAPK and NF-κB activation. The relevance of both pathways in promoting neuroprotection has been documented in a variety of paradigms, including excitotoxic stimuli (39, 45), amyloid beta-peptide toxicity (46, 47), and axotomy (37). Whether lack of JNK/SAPK and NF-κB response in ALS motor neurons is a primary or a secondary defect remains to be established.

The ISEL technique failed to detect DNA fragmentation suggestive of apoptotic cell death in ALS spinal cord. This is in agreement with previous findings by us (12) and by others (14). In our experience, ISEL is an extremely powerful technique for detecting apoptosis in animal models (56) and human biopsies (12, 57), where tissue preservation is optimal. In postmortem human tissue, however, ISEL is burdened with such widespread artefactual labeling due to autolytic DNA degradation that its usefulness becomes questionable (12). Studies on the expression of apoptosis-related proteases will hopefully represent a valid alternative to ISEL (57). Although activation of JNK/SAPKs, c-Jun and NF-κB has been observed during apoptosis in various experimental cell systems (16, 17, 29–31, 58), leading to the assumption that these proteins might be also involved in the apoptotic cascade, none of the ISEL-, JNK/SAPK-, c-Jun-, or NF-κB-labeled cells in our cases displayed an apoptotic morphology. On this basis, we conclude that (a) apoptosis is unlikely to play a major role in ALS; and (b) JNK/SAPK, c-Jun, and NF-κB do not appear to be involved in an apoptotic cascade in ALS.

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