NF-κB deficit in spinal motoneurons in patients with sporadic amyotrophic lateral sclerosis – a pilot study

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DOI: 10.5114/fn.2015.56551

Abstract
Amyotrophic lateral sclerosis (ALS) is a fatal incurable neurodegenerative disease whose etiology is unknown and pathogenesis is still not fully understood. A great majority of its cases are sporadic. Clinical ALS signs are caused by damage and dying-out of the lower and upper motor neurons. This study was aimed at identifying possible sporadic ALS-associated aberrations in the spinal cord expression of the transcription nuclear factor κ light-chain-enhancer of activated B cells (NF-κB). NF-κB is widely distributed among various cell types, including those specific for the central nervous system (CNS), and is involved in the control of many physiological and pathological processes, including, inter alia, inflammatory response, proliferation, angiogenesis, and cell survival and death. It is constitutively expressed and its inactive form resides in the cytoplasm. After activation, it enters the cell nucleus and promotes the transcription of target genes. NF-κB is a dimer and its most common form is a heterodimer made of subunits p50 and p65. In this study, we estimated and compared by immunohistochemical means the contents of these subunits in spinal cord motoneurons in a few archival cases of sporadic ALS of varying disease duration and the respective age-matched control cases with no CNS pathology. The major goal of the study was to seek possible changes in the expression of these proteins in the course of the disease. The control cases showed a strong expression of both p50 and p65 in spinal cord motoneurons, with both cytoplasmic and nuclear localization. In contrast, the ALS cases studies revealed a considerably lower and varying intensity of specific immunohistochemical staining for the two subunits, which suggested an increased deficit of their expression linked to longer disease duration. Moreover, there was an apparent shift toward mostly cytoplasmic localization of the two subunits. These preliminary data suggest that the changes in the expression of these NF-κB subunits may be involved in pathogenesis of sporadic ALS.

Key words: amyotrophic lateral sclerosis, motoneuron, neurodegeneration, NF-κB, spinal cord.

Introduction
Nuclear factor κ light-chain-enhancer of activated B cells (nuclear factor κB – NF-κB) is a protein complex functioning as a transcription factor. It has been discovered in 1986 in mouse lymphocytes B as a constitutively expressed complex that binds to the promoter of the gene encoding the light chain κ of immu-
noglobulins and is indispensable for the expression of the gene [41]. Inactive NF-κB resides in the cytoplasm, but enters the cell nucleus upon activation, binds to the so-called κB elements located in the promoter region of target genes and activates expression of the genes [27]. Extensive studies on NF-κB have revealed that it is widely distributed in various cell types in both the vertebrates and invertebrates and takes part in many cellular processes. Its action is closely connected to multiple intracellular signaling pathways ending in the synthesis of proteins linked to: 1) responses to a variety of stimuli, including, but not limited to, ischemia, free radical stress, cytokine and UV actions, and some antigens [5,11,12,36,45], 2) oncogenesis, regulation of inflammatory processes and responses to infections [21,22], and 3) processes related to learning and memory as well as synaptic plasticity [2]. Studies performed during the last decade have extended the list of NF-κB commitments with the processes linked to angiogenesis and cell proliferation, survival and death (including roles in both the prevention and promotion of apoptosis) [32,43]; most recently, NF-κB was also credited with a role in the pathogenesis of a growing number of neurodegenerative diseases [6].

NF-κB acts as a homo- or heterodimer of members of the Rel family that includes five proteins: p65 (RelA), RelB, c-Rel, p50 and p52 that are divided between two sub-families. One of the latter comprises p65, RelB and c-Rel, which all possess a transcription activation domain and due to this characteristic are able of activating NF-κB target genes. The other sub-family comprises p50 and p52 that originate from their respective precursors p100 and p105 and are devoid of this domain. All the Rel proteins possess the evolutionary highly conserved Rel homology domain. This domain is responsible for subunit dimerization and binding to DNA, which allows activation of the target genes [15], and encompasses a nuclear localization sequence that enables the Rel proteins entering the cell nucleus [32]. In the inactive state, the nuclear localization sequences are blocked by the Rel homology domain-bound inhibitory proteins of the κB family. Activation of NF-κB relies on phosphorylation of the respective κB inhibitor that is next subject to ubiquitination and proteosomal degradation. The inhibitor-free dimeric NF-κB translocates then to the nucleus [15].

In mammalian cells, the most common isoform of NF-κB is the p50-p65 heterodimer [32]. In the central nervous system (CNS), this isoform has been identified in the Schwann cells [7], astrocytes [44], microglia [35] and neurons [14,17,18,26,29,40]. The p65 subunit is considered the most important NF-κB subunit in the nervous system [26,27]. NF-κB plays an important role in multiple physiological processes in the nervous system both during the ontogeny and after reaching maturity. It is well-known that this factor is responsible both for the control of apoptosis [31,40] and the promotion of the survival of nervous system cells [23]. This duality in NF-κB action is the consequence of a wide spectrum of the targets of this transcription factor, which includes both some pro-apoptotic (e.g., Bax) and anti-apoptotic genes (e.g., bcl-2). An NF-κB target is also the Survival Motor Neuron (SMN) gene that plays an essential role in motoneuron function and survival.

Amyotrophic lateral sclerosis (ALS) is a fatal incurable neurodegenerative disease with relatively fast clinical course. Its etiology is unknown and pathogenesis is far from fully elucidated. Its progress and the fatal outcome rely on the damage and dying-out of the neurons of the spinal cord anterior horns, motor nuclei of the cranial nerves within the brain stem as well as motoneurons in the cerebral cortex, that is, of the lower and the upper motor neuron [20,34]. About 90% of all ALS cases are sporadic (sALS). The remaining cases represent the so-called familial ALS (fALS), a minor subset of which are linked to identifiable heritable genetic damage. Our earlier investigation has revealed a probable role of aberration(s) in the expression of the SMN complex proteins, in sALS pathogenesis. This is because we found a major decrease in gemin 2 level correlating with sALS duration [37]. The present study was aimed at identifying possible aberrations in the expression of the key transcription factor NF-κB subunits p50 and p65 in the spinal cord motoneurons of sALS patients with differing duration of the disease.

Material and methods

The study material included archival paraffin blocks with spinal cord samples harvested from the cervical enlargements (C4-C8 level) of four patients from 52 to 74 years of age, who died 1-8 years after the clinical onset of sALS. Before death, all the patients revealed lower motor neuron damage with tetraparesis and bulbar syndrome. The controls comprised spinal cords from two age-matched patients who died from CNS-unrelated causes and with no
record of neurological problems. Basic clinical characteristics of the cases and estimation of motoneuron loss in the spinal cord anterior horns in the studied samples are shown in Table I.

All experiments involving the human material were in agreement with the respective ethical principles as stipulated in the Helsinki Declaration and with the current law of Poland regarding the use of human organs and tissues in research. The study protocol has been filed with the Bioethics Committee of the Medical University of Warsaw. The Committee has raised no objection, and – because of the archival nature of the involved human tissue-containing material, full anonymization of the samples and the retrospective nature of the study – has waived the need for consent from the next of kin of the donors (Permit No. AKBE/20/14).

The paraffin blocks with the formalin-fixed spinal cord samples were cut transversely into 8 µm-thick sections, deparaffinized and rehydrated by routine procedures and then subjected to hematoxylin-eosin or cresyl violet staining or immunohistochemical labelings for p50 or p65 subunit of NF-κB. Immunohistochemistry was performed by the avidin-biotin-peroxidase method. Briefly, the rehydrated spinal cord slices were microwaved (3 × 10 min, in 10 mM citrate buffer pH 6.0) for antigen retrieval, and then were stained with primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) against p50 (cat. no. sc-114; dilution 1 : 250) or p65 (cat. no. sc-109; dil. 1 : 250). Next, the rehydrated spinal cord slices were microwaved (3 × 10 min, in 10 mM citrate buffer pH 6.0) for antigen retrieval, and then were stained with primary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) against p50 (cat. no. sc-114; dilution 1 : 250) or p65 (cat. no. sc-109; dil. 1 : 250). Next, the samples were incubated with biotinylated goat F(ab)2 fragment of anti-rabbit IgG (Beckman Coulter, cat. no. PN IM0830; dil. 1 : 1500), and consequently with streptavidin-horseradish peroxidase solution. The final immune complexes were developed with diaminobenzidine as the chromogen and assessed in a light microscope (Nikon, Japan) equipped with a model CCD camera (Nikon) and a computerized image analyzer system. Specificity of the reaction was tested using a negative control procedure (with primary antibodies omitted); the test showed no immunostaining. The intensities of specific p50 and p65 immunoreactivities were assessed in the respective slices (4–6 slices per case), in all visible spinal cord motoneurons, employing an 8-bit scale (256 levels of gray). The gray scale levels for p50 immunoreactivity from all the analyzed slices from a given case were next averaged and used as one observation for statistical analysis; the same was done for the respective p65 immunoreactivity data. The sALS cases analyzed were divided between two groups: the group with short (1-year, n = 2) and the group with long (2- or 8-year, n = 2) course of the disease; the two control cases with no ALS comprised the control group. The quantitative immunostaining data were subject to a one-way ANOVA with group as the between-subject factor followed by the Tukey test.

Cell morphology in the studied samples was assessed in a separate series of the spinal cord samples that were subject to routine hematoxylin-eosin (H&E) and cresyl violet stainings, air-dried, coverslipped using Permount™ and then examined in the aforementioned computerized Nikon light microscopy system.

Results

Spinal cord sections from controls showed no symptoms of degeneration, and the motoneurons were of normal morphology (Fig. 1). The sections from sALS cases, both those with short (1 year) and longer (2 and 8 years) disease history, on the contrary, revealed obvious aberrations in the mor-

<table>
<thead>
<tr>
<th>Group</th>
<th>Case No.</th>
<th>Gender</th>
<th>Age (years)</th>
<th>Cause of death</th>
<th>sALS duration (years)</th>
<th>Post-mortem time (h)</th>
<th>Motoneuron loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1</td>
<td>F</td>
<td>60</td>
<td>Digestive system hemorrhage</td>
<td>–</td>
<td>41</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>67</td>
<td>Heart infarction</td>
<td>–</td>
<td>14</td>
<td>–</td>
</tr>
<tr>
<td>sALS</td>
<td>1</td>
<td>M</td>
<td>52</td>
<td>sALS</td>
<td>1</td>
<td>30</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>F</td>
<td>59</td>
<td>sALS</td>
<td>1</td>
<td>22</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>M</td>
<td>73</td>
<td>sALS</td>
<td>2</td>
<td>12</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>M</td>
<td>74</td>
<td>sALS</td>
<td>8</td>
<td>15</td>
<td>Extensive</td>
</tr>
</tbody>
</table>
morphology of the surviving motoneurons: changes in both the shape and size (shrinkage), hyperchromatosis, and (typical of damaged neurons) tigrolysis of intensity that showed a great variability with regard both to individual case and cell (Fig. 2). The two sALS cases with 1-year history of the disease showed the presence of few tigrolysis-affected motoneurons; the numbers of these cells were considerably higher in the sALS cases with longer disease duration. Interestingly, the spinal cord sections from the former showed also the presence of few motoneurons with enlarged Nissl bodies, which might be related to a transient reinervation that occurs during the initial stage of the disease and may represent a compensation for degeneration of the other motoneurons (Fig. 2). Compared to the controls, the sections from sALS cases showed also clearly lower total motoneuron numbers, but no signs of apoptosis. This difference was rather small for the cases with short disease history, but was much higher in those with longer disease duration (see Fig. 1 and Table I).

For both p50 and p65, immunohistochemistry revealed a moderate cytoplasmic staining in non-neuronal cells, and an intense staining of the cytoplasm and even more intense staining of the nuclei of motoneurons in the controls; the apparent immunoreactivity was higher for p50 (Figs. 3 and 4). The motoneurons in spinal cord sections from sALS cases showed lower immunoreactivity levels for both p50 (Fig. 3) and p65 (Fig. 4). The sections from sALS cases compared to those from controls contained also considerably lower numbers of motoneurons with p50- or p65-positive nuclei. This difference was particularly evident in the two sALS cases with
Motoneuronal NF-κB deficit in sporadic ALS

Control 1  Case 1: sALS, 1 year duration  Case 4: sALS, 8 years duration

Fig. 2. Cresyl violet staining of spinal cord anterior horn sections from a control and selected sporadic amyotrophic lateral sclerosis (sALS) cases. The microphotograph of a section from a control (far left) shows a motoneuron of normal morphology, including typical-looking Nissl granules. The two middle microphotographs of a section from an sALS case with 1-year history show an enlargement (right) and decay (left) of Nissl bodies. The microphotograph of a section from an sALS case with 8-year history reveals extensive tigrolysis.

longer disease history. In the two cases with 1-year sALS duration there were a few motoneurons with p50- or p65-positive nucleus in each section analyzed. The remaining motoneurons showed p50 or p65 positivity only in the cytoplasm. The other two sALS cases (with longer disease history) revealed even lower numbers of motoneurons. Notably, the surviving motoneurons showed a weak cytoplasmic staining, and only a minor fraction of these (a few in the case with 2-year, and only one in the case with 8-year ALS history, in all the studied sections combined) showed the presence of p50 or p65 in the nucleus.

Interestingly, the non-neuronal cells present in the spinal cord anterior horns sections showed heavy immunolabeling for both p50 and p65 in all sALS cases studied (Figs. 3 and 4). Many of these cells revealed nuclear localization of the NF-κB subunits.

Statistical analysis revealed significantly lower intensities of both the p50 and p65 immunoreactivities in the sALS cases with either 1-year or longer disease duration compared to the controls, while there was no statistically significant difference in this respect between the two sALS subsets (Table II). There was a high positive linear correlation between the p50 and p65 staining intensities as assessed across all the cases (sALS and controls combined) included in this study (n = 6; Pearson’s correlation coefficient: R = 0.97, p = 0.0014). This correlation was in line with the presumed co-expression of the two NF-κB subunits in the same cells and their presumed heterodimerization.

Discussion

A large body of evidence indicates that NF-κB-mediated signaling plays an important role in neuron survival in many pathological entities, for review see [19,32]. The decrease in NF-κB expression and the shift toward its prevailing cytoplasmic localization associated with increased duration of the disease in our material of sALS strongly suggests a failing function of this transcription-regulating factor. This result may put in doubt the presumed importance of NF-κB for the processes that promote either the death or the survival of the analyzed cells. However, the absence of this factor in the nuclear compartment may indirectly contribute to the deficient neuroprotection, e.g. due to a diminished NF-κB-dependent expression of the anti-apoptotic Bcl-2 gene [27].

The link between motoneuron death and function of anti- (e.g., Bcl-2) and proapoptotic proteins (e.g., Bax) is supported by a steadily growing number of studies [1,10,25,33,39,42].

Another important target gene of NF-κB is SMN that plays an essential role in both motoneuron survival and function. However, our earlier studies have revealed similar SMN immunoreactivity in sALS autopsy cases greatly differing in disease duration. The only exception was a single case with 8-year history of ALS with prominent symptoms of neurodegeneration in the few surviving motoneurons and
Fig. 3. NF-κB p50 subunit immunoreactivity in the anterior horn of spinal cords from controls and sporadic amyotrophic lateral sclerosis (sALS) cases. The sections from controls (top panel) show the presence of heavily stained motoneurons (long arrows) with both cytoplasmic and nuclear p50 staining. The sections from sALS cases (middle and bottom panels) reveal little or no p50 staining, particularly of cell nuclei; white arrowheads point to glial cells.
**Fig. 4.** NF-κB p65 subunit immunoreactivity in the anterior horn of spinal cords from control and sporadic amyotrophic lateral sclerosis (sALS) cases. The sections from controls (top panel) show the presence of intensively stained motoneurons (long arrows) with both cytoplasmic and nuclear p65 staining. The sections from sALS cases (middle and bottom panels) demonstrate little or no p65 labeling intensity, particularly of cell nuclei; white arrowheads point to glial cells.
clearly lowered SMN level; the latter was obviously related to the poor condition of the cells [37].

The continuing SMN expression at the absence of NF-κB in the nucleus suggests that the expression under these conditions is controlled by different transcription factor(s); it has been shown that SMN is a target gene also for Elk-1 [9] and CREB [24]. However, the functionality of the SMN protein complex-dependent motoneuron survival pathway is in serious doubt. This is because of the evidence indicating a deficient production in sALS of gemin 2 that is a key subunit responsible for SMN binding with the other subunits of the SMN protein complex [37].

The actual role of NF-κB in the ALS-related neurodegeneration is presently under intense scrutiny, but the existing data are equivocal. Studies in transgenic mice overexpressing a mutated form of human SOD-1 have shown that blocking apoptosis by pharmacological induction of NF-κB in motoneurons prolongs survival of the cells [38]. Surprisingly, a neuroprotective effect on spinal cord motoneurons was also achieved in a mouse ALS model with NF-κB inhibition [46]. The results of the present study suggest that the progress of ALS may linked to a dysfunction in the mechanisms governing NF-κB activation.

Some studies in a rat model of traumatic CNS injury have shown that blockade of the NF-κB-mediated signaling pathway in astrocytes can exert a neuroprotective effect [30]. However, an attempt to use the same strategy for motoneuron protection in a mouse model of ALS has failed. It appeared that the pathomechanism of the neuronal damage in this model was much more complex and the inhibition of but one out of a number of pathways contributing to motoneuron damage was insufficient for an effective neuroprotection [8].

**Conclusions**

The role of NF-κB in sALS remains far from elucidated. The apparent decline in its immunoreactivity in spinal cord motoneurons with increasing duration of the disease may result either from a diminished expression or from an increased turnover of the respective NF-κB subunits, or both. The major shift toward prevailing cytoplasmic localization of NF-κB suggests that there is also a considerable disruption of the machinery governing the activation of this transcription factor in the motoneurons. Both these phenomena may contribute to the pathogenesis of the neurodegeneration process in sALS, but this issue needs further studies.

An impaired NF-κB function in spinal cord motoneurons is most likely but one of multiple factors involved in ALS pathogenesis. The effect of perturbations affecting the function of this transcription factor in the motoneurons may also be related to its performance in the other cell types in the spinal cord. An important role in this regard may be played by enhanced NF-κB expression/activity in glial cells, which may result in a boosted production and release of factors with a potential detrimental action, e.g., of pro-inflammatory cytokines, excitotoxins and free radicals.
Acknowledgment

This study was supported by the National Centre for Research and Development Project NN 401 014640 and by statutory funds from the Mossakowski Medical Research Centre, Polish Academy of Science.

Disclosure

Authors report no conflict of interest.

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