Nervous immunity: A study on the role of complement system in neuronal degeneration and regeneration
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Chapter 8:
Toll-like receptor signaling in amyotrophic lateral sclerosis
spinal cord tissue

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Abstract

Increasing evidence indicates that inflammatory responses could play a critical role in the pathogenesis of motor neuron injury in amyotrophic lateral sclerosis (ALS). Recent findings have underlined the role of Toll-like receptors (TLRs) and the receptor for advanced glycation end products (RAGE) in the regulation of both innate and adaptive immunity in different pathologies associated with neuroinflammation. In the present study we investigated the expression and cellular distribution of TLR2, TLR4, RAGE and their endogenous ligand high mobility group box 1 (HMGB1) in the spinal cord of control (n=6) and sporadic ALS (n=12) patients. The immunohistochemical analysis of TLR2, TLR4 and RAGE showed increased expression in reactive glial cells in both gray (ventral horn) and white matter of ALS spinal cord. TLR2 was predominantly detected in cells of the microglia/macrophage lineage, whereas the TLR4 and RAGE was strongly expressed in astrocytes. Real-time quantitative PCR analysis confirmed the increased expression of both TLR2 and TLR4 and HMGB1 mRNA level in ALS patients. In ALS spinal cord, HMGB1 signal is increased in the cytoplasm of reactive glia, indicating a possible release of this molecule from glial cells. Our findings show increased expression of TLR2, TLR4, RAGE and HMGB1 in reactive glia in human ALS spinal cord, suggesting activation of the TLR/RAGE signaling pathways. The activation of these pathways may contribute to the progression of inflammation, resulting in motor neuron injury. In this context, future studies, using animal models, will be important to achieve a better understanding of these signaling pathways in ALS in view of the development of new therapeutic strategies.
Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by loss of motor neurons and extensive astrogliosis and microglial activation in motor cortex and spinal cord (Ince et al., 1998; Piao et al., 2003). Despite identification of disease-linked mutations the aetiology and pathogenesis of ALS are still elusive (Pasinelli and Brown, 2006; Vivekananda et al., 2008; Kwiatkowski et al., 2009). Recent experimental and clinical observations have suggested role for non-neuronal cells, including both microglia and astrocytes, indicating the involvement of both the innate and adaptive immune responses in ALS pathogenesis (Hall et al., 1998; Levine et al., 1999; McGeer and McGeer, 2002; Pasinelli and Brown, 2006). Focus has recently shifted to Toll-like receptor (TLR) signaling pathways in neurodegenerative disorders, including ALS (Letiembre et al., 2009). TLRs are evolutionarily conserved transmembrane glycoproteins forming the “sensors” of the innate immune system and mediating the sustained glial reactivity observed under pathological conditions (Kielian, 2006). In the absence of pathogens, TLR signaling can be activated by molecules, called damage associated molecular patterns (DAMPs) including the high mobility group box 1 (HMGB1) protein, released by the injured tissue (Bianchi and Manfredi, 2009). HMGB1 is a nearly ubiquitous chromatin component regulating transcription of different sets of genes, including proinflammatory genes (Pedrazzi et al., 2007; Mouri et al., 2008; Bianchi and Manfredi, 2009). However, HMGB1 can be passively released by necrotic cells and actively secreted by stimulated monocytes/macrophages and astrocytes (Scaffidi et al., 2002; Andersson et al., 2008; Bianchi, 2009; Hreggvidsdottir et al., 2009; Hayakawa et al., 2010; Maroso et al., 2010) binding to the receptor for advanced glycation end products (RAGE) and other receptors, including TLR2 and TLR4 (Scaffidi et al., 2002; Parker et al., 2004). Recent evidence suggests a critical role for the HMGB1-TLR4 pathway in the regulation of neuronal excitability and neurotoxicity (Kleen and Holmes, 2010) mediated by N-methyl-D-aspartic acid (NMDA) receptor activation involving a tyrosine phosphorylation of the MDA receptor regulatory subunit 2B that controls Ca\(^{2+}\) influx (Maroso et al., 2010). Interestingly, increased levels of TLR have been observed in mutant superoxide dismutase 1 (SOD1) mice, as compared to controls (Letiembre et al., 2009) and mutant SOD1 expression in ALS has been suggested to facilitate microglial neurotoxic inflammatory responses via TLR2 (Liu et al., 2009). In addition, it has recently been shown that mutant SOD1 binds to CD14, which is a co-receptor of TLR2 and TLR4, and that the microglial activation mediated by mutant SOD1 (G93A) can be attenuated using TLR2, TLR4 and CD14 blocking antibodies (Zhao et al., 2010).

A key challenge is to translate this information concerning the role of the innate immune system in ALS into the clinic. A helpful first step in this direction is represented by the evaluation of specific inflammatory pathways in human tissue and the identification of appropriate patient populations. Clinically and morphologically well characterized material from ALS patients provides a valuable source of information, which may guide future experimental studies aimed at the development of new therapeutic strategies. To determine whether the TLR/RAGE signaling pathways are involved in the inflammatory response in ALS, we investigated the expression patterns of TLR2, TLR4, RAGE and HMGB1 in normal and ALS spinal cord from patients with sporadic ALS and different disease duration. Our aim was to define the possible involvement of TLR/RAGE signaling in the pathophysiology of ALS and increase our knowledge on the role of inflammation in this disease.
Materials and methods

Subjects

Post-mortem material was obtained at autopsy from 12 sporadic ALS patients at the department of Pathology of the Academic Medical Center (University of Amsterdam). All patients fulfilled the diagnostic criteria for sporadic ALS (sALS; El Escorial criteria; Brooks et al., 2000), were reviewed independently by two neuropathologists and the diagnosis of ALS was confirmed according to the standard histopathological criteria (Ince et al., 1998; Piao et al., 2003). The group included six patients with rapid disease progression and short-term survival <18 month (ALS-st) and six patients with slow disease progression and long-term survival > 48 months (ALS-lt) (Table 1). All patients with ALS died for respiratory failure. The control spinal cord tissue was obtained from six patients (four males and two females) who had died from a non-neurological disease (cause of death: myocardial infarction, renal failure, pulmonary embolism). Both ALS and control patients included in the study displayed no signs of infection before death.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex</th>
<th>Age</th>
<th>Duration of disease (mon)</th>
<th>Clinical diagnosis</th>
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<td>1</td>
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<td>57</td>
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<tr>
<td>2</td>
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<td>96</td>
<td>sALS</td>
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<tr>
<td>Mean±SEM</td>
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<td></td>
<td>59.5±3.4</td>
<td>43.5±10.6</td>
</tr>
</tbody>
</table>

Table 1: Summary of clinical and neuropathological data of ALS and control patients. NC: normal controls; sALS: sporadic ALS.
Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue

Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes and approval was obtained from the relevant local ethical committees for medical research. All autopsies were performed within 12 h after death. After removal of the spinal cord, 0.5 cm thick slices, taken from the cervical (C7), thoracic (T4 and T8) and lumbar (L1) levels, were frozen and stored at -80°C; the remainder of the cord was fixed in 10% buffered formalin.

Tissue preparation

Paraffin-embedded tissue was sectioned at 6 µm and mounted on pre-coated glass slides (StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany). Representative sections of all specimens were processed for haematoxylin and eosin, Klüver-Barrera and Nissl stains. To define different cell populations we used serial sections stained with Nissl, astroglial and microglial markers (GFAP and HLA-DR, see below).

Frozen tissue stored at -80°C, was used for PCR analysis.

RNA isolation and real-time quantitative PCR analysis (RT-PCR)

For RNA isolation, frozen material was homogenized in Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA). After addition of 200 µg glycogen and 200 µl chloroform, the aqueous phase was isolated using Phase Lock tubes (Eppendorf, Hamburg, Germany). The concentration and purity of RNA were determined spectrophotometrically at 260/280 nm using a nanodrop spectrophotometer (Ocean Optics, Dunedin, FL, USA). 5 µg of total RNA was reverse-transcribed into cDNA using oligo dT primers. Real-time quantitative PCR analysis (RT-PCR) was performed in a LightCycler® 480 Real-Time PCR System (Roche-Applied-Science, Indianapolis, IN, USA) using RNA from human control spinal cord (n = 6) and spinal cord from patients with ALS (n = 12). The following PCR primers (Eurogentec, Belgium) were used: TLR2 (forward: tgatgcgtgcattctactc; reverse: cgcagctctcagatttaccc), TLR4 (forward: aatcccctgaggcatttagg; reverse: aatactctggatggggtttcc), RAGE (forward: aggaccagggaacctacagc; reverse: cctgatcctcccacagc), HMGB1 (forward: aagaccaccagatgcttcagt; reverse: tccgcttttgccatatcttc), TATA box-binding protein (TBP; forward: caggagccaagagtgaagaac; reverse: aggaaataactctggctcataactc), hypoxanthine phosphoribosyl transferase (HPRT; forward: tggcgtcgtcgtgattagtgatg; reverse: tgtaatccagcaggtacgca) and elongation factor 1 alpha (EF1α; forward: atccaccttgggtgctcctt; reverse: cgcaactgtctgtcatcatac). The data were quantified using the LinRegPCR program as described previously (Ramakers et al., 2003). The amount of each specific product was divided by amount of the reference genes (TPB, HTRP, EF1α).

In situ hybridization

In situ hybridization for human TLR2 and TLR4 was performed using a 5= fluoroescein labeled 19mer antisense oligonucleotide (Ribotask ApS, Odense, Denmark) containing Locked Nucleic Acid and 2=OMe RNA moieties (TLR2: 5=FAM-TagCucTguAgaTcuGaaG; TLR4: 5=FAM-TucTuuAcuAgCtcTucC; capitals indicate locked nucleic acid [LNA], lower case indicates 2= O-methyl [OME] RNA). The hybridizations were performed at 59°C on 6 µm sections of paraffin embedded material as described previously (Budde et al., 2008).

Immunocytochemistry

Glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000), vimentin (mouse clone V9, DAKO; 1:400), neuronal nuclear protein (NeuN; mouse clone...
MAB377, IgG1; Chemicon, Temecula, CA, USA; 1:1000), major histocompatibility complex (MHC) class II antigen (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO, Glostrup, Denmark; 1:400) and CD68 (mouse clone PG-M1, DAKO; 1:200) were used in the routine immunocytochemical analysis of ALS specimens.

For the detection of TLR4 we used a rabbit polyclonal antibody (Santa Cruz, sc10741; CA, USA; 1:20), for TLR2 a polyclonal goat (R&D systems; 1:100), for RAGE goat anti-RAGE antibody (AGE 001; Biologo, Kronshagen, Germany; 1:200), and for HMGB1 rabbit polyclonal antibody (Abcam, Cambridge, UK). Single-label immunocytochemistry was performed as previously described (Aronica et al., 2001, 2003) with the Powervision kit (Immunologic, Duiven, The Netherlands) and 3,3-diaminobenzidine as chromogen. For double-label immunocytochemistry with anti-TLR2, TLR4, RAGE, HMGB1 and anti-GFAP or anti-HLA-DR, the chromogens used were Vector Blue- and Nova RED-substrate (Vector Laboratories, Burligame, CA, USA).

**Evaluation of immunostaining**

All labeled tissue sections were evaluated by two independent observers blind to clinical data, for the presence or absence of various histopathological parameters and specific immunoreactivity (IR) for the different markers. The intensity of TLR4, TLR2, RAGE and HMGB1 immunoreactive staining was evaluated using a scale of 0–3 (0: –, no; 1: ± weak; 2: +, moderate; 3: ++, strong IR). The approximate proportion of cells showing TLR and RAGE IR [(1) single to 10%; (2) 11–50%; (3) >50%] was also scored to give information about the relative number ('frequency' score) of positive cells within the ALS specimens. As previously reported (Vandeputte et al., 2002; Ravizza et al., 2006), the product of these two values (intensity and frequency scores) gave the total score shown in Fig. 7. Numbers of positive cells in HMGB1 double staining were quantified as previously described (Maroso et al., 2010). Briefly, two representative adjacent non-overlapping fields of cervical spinal cord white matter were captured (magnification 40x) and digitized using a laser scanning confocal microscope (Leica SP2, Wetzlar, Germany). The total number of GFAP- and HLA-DR (human leukocyte antigen- DP, DQ, DR)—positive cells and those showing nuclear or extra-nuclear HMGB1 staining was counted. The expression of the MHC class II glycoprotein, HLA-DR is prominently upregulated when microglia becomes reactive. This has been observed in ALS and different other pathological conditions (Overmyer et al., 1999; McGeer and McGeer, 2002, 2003). Only activated HLA-DR cells expressing HMGB1 were counted since the morphology of resting or weakly activated microglia (small cell bodies with extensive ramifications and low HLD-DR expression) did not allow an accurate counting.

**Statistical analysis**

Statistical analyses were performed with SPSS for Windows (SPSS 11.5, SPSS Inc., Chicago, IL, USA) using a non-parametric Kruskal-Wallis test followed by the Dunn’s post-hoc test. The possible correlation between IR score and different clinical variables (age, gender, duration and site of onset) was analyzed using Pearson’s correlation coefficient (p). P < 0.05 was considered significant.
Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue

Results

Case material

The clinical and neuropathological characteristics of the subjects are summarized in Table 1. There were no significant differences between the ALS and normal control groups with respect to post-mortem interval or duration of storage. None of the control patients had confounding neurological or neuropathological abnormalities.

TLR2 in ALS spinal cord

Quantitative analysis of TLR2 mRNA expression in ALS spinal cord

Increased TLR2 mRNA expression was observed in both ALS-st and ALS-lt patients, compared to control spinal cord by RT-PCR (Fig. 1A; \( P < 0.05 \)). No significant difference in TLR2 mRNA expression was observed between ALS-st and ALS-lt (\( P > 0.05 \)).

TLR2 cellular distribution in ALS spinal cord

Fig. 2A–F shows TLR2 protein expression in a representative normal and ALS cervical spinal cord ventral horn and white matter. Control motor neurons and the large majority of resting glial cells did not express detectable levels of TLR2 IR (Figs. 2A, B and 7A). In all ALS cases, the intensity of TLR2 appeared to be increased in cells with typical glial morphology in both white matter and gray matter (Figs. 2C–F and 7A). Double labeling demonstrated TLR2 expression in cells of the microglial/macrophage lineage (HLA-DR positive cells; insets b, c in Fig. 2F) but not reactive astrocytes (not shown). Fig. 7A shows higher TLR2 glial IR (total) score compared to controls in both ALS-st and ALS-lt. Cellular distribution of TLR2 mRNA studied by in situ hybridization showed positivity in glial cells in ALS specimens, but not in resting glial cells in control spinal cord, confirming the protein expression pattern (not shown).

Figure 1: RT-PCR of TLR2, TLR4 and HMGB1 expression in control and ALS spinal cord. Samples were analyzed in duplicate, corrected for the expression levels of reference genes. Expression levels in st-ALS (patients with rapid disease progression and short-term survival <18 mon; \( n=6 \)) and lt-ALS (patients with slow disease progression 7–18 mon, \( n=6 \); and long-term survival <48–96 mon, \( n=6 \)) were compared to levels in autopsy control spinal cord (\( n=6 \)). TLR2 (A) and TLR4 (B) mRNA levels were significantly increased in st-ALS and lt-ALS specimens compared to controls. HMGB1 (C) mRNA levels were significantly increased in lt-ALS specimens compared to controls. There were no significant differences in TLR2 and TLR4 and HMGB1 between st-ALS and lt-ALS. The error bars represent SEM and * represents \( P < 0.05 \).
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Figure 2: Cellular distribution of TLR2 in the cervical spinal cord of control (A, B) and ALS (C–F). Panels (A, B): representative photomicrographs from Lt-ALS of immunohistochemical staining for TLR2 in control cervical spinal cord showing no detectable expression in neurons and in the large majority of glial cells in both ventral horn (VH) and white matter (WM); only occasionally few positive cells are observed around blood vessels (inset in B, arrow). Panels (C–F): representative photomicrographs of immunohistochemical staining for TLR2 in ALS cervical spinal cord. A substantial increase in TLR2 immunoreactivity (IR) is observed in ALS VH (C, D) and WM (E, F). (C, E): low magnification of VH (C) and WM (E) with TLR2 IR. (D): high magnification of VH showing stained glial cells (with the morphology of microglial cells; arrows) surrounding residual motor neurons. (F): high magnification of WM (lateral corticospinal tract) showing TLR2 positive glial cells (arrows); inset (a) in (F) shows TLR2 positive cells around a blood vessel. Insets (b, c) in (F) show colocalization (purple) of TLR2 (red) with HLA-DR (blue) in cells of the microglia/macrophage lineage. Sections are counterstained with hematoxylin. Scale bars: (A–C, E): 140 µm. (D, F): 40 µm.

TLR4 in ALS spinal cord

Quantitative analysis of TLR4 mRNA expression in ALS spinal cord

Increased TLR4 mRNA expression was observed in both ALS-st and ALS-lt patients, compared to control spinal cord by RT-PCR (Fig. 1B; P < 0.05). No significant difference in TLR4 mRNA expression was observed between ALS-st and ALS-lt (P > 0.05).
Figure 3: Cellular distribution of TLR4 immunoreactivity (IR) in the cervical spinal cord of control (A, B) and ALS (C–F). Panels (A, B): representative photomicrographs of immunohistochemical staining for TLR4 in the control cervical spinal cord showing variable immunoreactivity (IR) in motor neurons (arrows) and no detectable or low expression in the large majority of glial cells in both ventral horn (VH, A; inset in A) and white matter (WM); inset in (B) shows TLR4 IR in a blood vessel (endothelial IR). Panels (C–F): representative photomicrographs of immunohistochemical staining for TLR4 in ALS cervical spinal cord. A substantial increase in TLR4 IR is observed in ALS VH (C, D) and WM (E, F). (C, E): low magnification of VH (C) and WM (E) with TLR4 IR. (D): high magnification of VH showing stained glial cells (arrow-heads) surrounding a residual TLR4 positive motor neuron (arrow). Inset (a) in (D) shows strongly stained reactive astrocytes. Inset (b) in (D) shows: colocalization (purple) of TLR4 (red) with GFAP (blue) in astrocytes. Inset (c) in (D) shows absence of colocalization of TLR4 (red) with HLA-DR (blue) in cells of the microglia/macrophage lineage. (F): high magnification of WM (lateral corticospinal tract) showing TLR4 positive reactive glial cells (arrows); inset in (F) shows a TLR4 positive astrocyte. Sections are counterstained with Hematoxylin. Scale bars: (A–C, E): 140 μm. (D, F): 40 μm.

TLR4 cellular distribution in ALS spinal cord

Fig. 3A–F shows TLR4 protein expression in a representative normal and ALS cervical spinal cord ventral horn and white matter. Moderate TLR4 IR was detected in control motor neurons, but not in the large majority of resting glial cells (Figs. 3A, B and 7B). In all ALS cases, the intensity of TLR4 appeared to be increased in cells with typical glial morphology in both white matter and gray matter (Figs. 3C–F and 7B). Double labeling demonstrated TLR4 expression in reactive astrocytes (GFAP positive cells; inset b in Fig. 3D), but not cells of the microglial/macrophage lineage (HLA-DR positive cells) (inset c in Fig. 3D). Fig. 7B shows higher...
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TLR4 glial IR (total) score compared to controls in both ALS-st and ALS-lt. Cellular distribution of TLR4 mRNA studied by *in situ* hybridization showed positivity in glial cells in ALS specimens, but not in resting glial cells, confirming the protein expression pattern (not shown).

**RAGE in ALS spinal cord**

*Quantitative analysis of RAGE mRNA expression in ALS spinal cord*

No significant difference in RAGE mRNA expression was observed by RT-PCR in both ALS-st and ALS-lt patients, compared to control spinal cord, as well as between the two ALS groups (P > 0.05; not shown).

*RAGE cellular distribution in ALS spinal cord*

Fig. 4A–F shows RAGE protein expression in a representative normal and ALS cervical spinal cord ventral horn and white matter. RAGE IR was detected in control motor neurons, but not in a large majority of resting glial cells (Figs. 4A, B and 7C). In all ALS cases, the expression pattern remained unchanged in the residual neurons, but the intensity of RAGE appeared to be increased in cells with typical glia morphology in both white matter and gray matter (Figs. 4C–F and 7C). Double labeling demonstrated RAGE expression in reactive astrocytes (GFAP positive cells; inset a in Fig. 4D), as well as in cells of the microglial/macrophage lineage (HLA-DR positive cells; inset b in Fig. 4D). Fig. 7C shows higher RAGE glial IR (total) score compared to controls in both ALS-st and ALS-lt.

**HMGB1 in ALS spinal cord**

*Quantitative analysis of HMGB1 mRNA expression in ALS spinal cord*

Significant increase of HMGB1 mRNA expression was observed by RT-PCR in ALS-lt patients, compared to control spinal cord (Fig. 1C; P < 0.05), whereas ALS-st shows a trend in the same direction (P=0.055). No significant difference in HMGB1 mRNA expression was observed between ALS-st and ALS-lt (P > 0.05).

**HMGB1 cellular distribution in ALS spinal cord**

Fig. 5A–G shows HMGB1 protein expression in control and ALS cervical spinal cord ventral horn and white matter. In human control spinal cord variable IR was detected in the cytoplasm of motor neurons, whereas resting glial cells showed nuclear expression (Fig. 5A, B). In ALS cases, residual motor neurons displayed variable nuclear/cytoplasmic IR, whereas cytoplasmic HMGB1 staining was substantially increased in glial cells (Figs. 5C–G and 7D). Double labeling confirmed the HMGB1 expression in glial cells, including both astrocytes and activated microglial cells (Fig. 5F, G, insets). Quantification of HMGB1 positive glial cells confirmed the increased cytoplasmic HMGB1 staining in ALS, compared to control spinal cord specimens (Fig. 6A, B).
Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue

Figure 4: Cellular distribution of RAGE in the cervical spinal cord of control (A, B) and ALS (C–F). Panel (A, B): representative photomicrographs of immunohistochemical staining for RAGE in the control cervical spinal cord showing light immunoreactivity (IR) in motor neurons (arrows) and no detectable or low expression in the large majority of glial cells in both ventral horn (VH; A; inset in A) and white matter (WM; inset in B). Panels (C–F): representative photomicrographs of immunohistochemical staining for RAGE in ALS cervical spinal cord. A substantial increase in RAGE IR is observed in ALS VH (C, D) and WM (E, F). (C, E): VH (C) and WM (E) with RAGE IR in residual motor neurons (arrows in C) and in glial cells (arrow-heads). (D): high magnification of VH showing stained glial cells (arrow-heads) surrounding residual TLR4 positive motor neurons (arrows). Inset (a) in (D) shows: colocalization (purple) of RAGE (red) with GFAP (blue) in astrocytes. Inset (b) in (D) shows absence of colocalization of RAGE (red) with HLA-DR (blue) in cells of the microglia/macrophage lineage. (E–F): WM (lateral corticospinal tract) showing and RAGE positive reactive glial cells (arrows); Scale bars: (A–C): 140 µm; (D, F): 40 µm; (E): 100 µm.

No significant change in the number of motor neurons showing nuclear and/or cytoplasmic staining was found in ALS spinal cord (data not shown). No correlation was found between the increased cytoplasmic glial HMGB1 staining and clinical variables. Fig. 7D shows higher HMGB1 glial IR (total) score compared to controls in both ALS-st and ALS-lt.
Figure 5: Distribution of HMGB1 immunoreactivity (IR) in the cervical spinal cord of control (A, B) and ALS (C–G). Panels (A, B): representative photomicrographs of ALS of immunohistochemical staining for HMGB1 in the control cervical spinal cord (A, ventral horn, VH; and B white matter, WM) showing variable cytoplasmic IR in motor neurons (arrows in A) and nuclear IR in glial cells (arrowheads in A; arrows in B). Panels (C–F): representative photomicrographs of immunohistochemical staining for HMGB1 in ALS cervical spinal cord. (C, D): ALS VH showing strong nuclear expression in glial cells with numerous immunoreactive processes (arrows in C; inset in C; arrowheads in D; inset in D); variable expression is observed in motor neurons: panel (D) (arrow) shows a motor neuron with prominent nuclear and cytoplasmic staining; inset in (D) shows a negative motor neuron. Panel (E): ALS WM showing nuclear and cytoplasmic IR in reactive glial cells (arrows). Panels (F, G): high magnification photomicrographs of positive cells in WM (arrow in G indicates immunoreactive glial processes). Inset in (F): colocalization (purple) of HMGB1 (red) with GFAP (blue) in astrocytes. Inset in (G): colocalization (purple) of HMGB1 (red) with HLA-DR (blue) in cells of the microglia/macrophage lineage. Sections are counterstained with Hematoxylin. Scale bars: (A): 80 µm. (B–E): 40 µm. (F): 25 µm; (G): 10 µm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Discussion

In the present report we studied the involvement of inflammation in ALS focusing on innate immune mechanisms such as the TLR signaling pathway (Bsibsi et al., 2002; Aravalli et al., 2007; Crack and Bray, 2007; Andersson et al., 2008; Drexler and Foxwell, 2010; Maroso et al., 2010). An up-regulation of TLR2, TLR4 and HMGB1 expression was demonstrated in specimens of patients with sALS, thus providing direct evidence of a chronic inflammatory state involving the TLR/RAGE pathways in human ALS.
Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue

**Figure 6**: Quantification of HMGB1-positive glial cells in control and ALS specimens. (A): astrocytes. (B): microglia/macrophages. ALS-st, patients with rapid disease progression and short-term survival <18 mon; ALS-lt, patients with slow disease progression and long-term survival >48 mon; nd, not detectable. Extra-nuclear staining: * \( P < 0.05 \) versus control, Kruskal-Wallis test followed by the Dunn’s post-hoc test.

**TLR2 and TLR4 expression in ALS spinal cord**

Consistent up-regulation of TLR2 and TLR4 mRNA and protein was observed in ALS spinal cord, compared to control tissue showing, furthermore, a differential cellular distribution for the two proteins. TLR2 protein was observed in activated microglial cells in both gray (ventral horn) and white matter of ALS spinal cord. Previous *in vitro* studies demonstrated constitutive expression of TLR2 and an up-regulation of the receptor expression after activation (for review see Kielian, 2006). TLR2 in microglia may mediate several pathways leading to either neuroprotective or neurotoxic phenotypes under different experimental conditions (Babcock et al., 2006; Kielian, 2006; Aravalli et al., 2007; Mallard et al., 2009). Interestingly, activation of cells of the microglia/macrophage lineage and induction of different inflammatory pathways have been described in human ALS tissue, (Troost et al., 1990; Henkel et al., 2004; Moisse and Strong, 2006; Sta et al., 2011). Our observations support the role of TLR2 signaling pathway in regulating microglia function in human ALS. The expression pattern of TLR4 differed from TLR2, showing a prominent expression in both glial and neuronal cells. Whereas, reactive astrocytes present within gray (ventral horn) and white matter of ALS spinal cord express TLR4, its expression in astrocytes *in vitro* appears controversial (for review see Kielian, 2006; Crack and Bray, 2007). Some studies were unable to detect TLR4 expression (Farina et al., 2005; Kielian, 2006), whereas others have shown a constitutive expression of TLR4 in astrocytes and an up-regulation following activation (Bsibsi et al., 2002; Bowman et al., 2003; Carpentier et al., 2005). These discrepancies may reflect species differences, as well as differences in culture conditions (Kielian, 2006). Moreover, glial TLR expression may be influenced by pro-inflammatory cytokines (such as IL-1β) or molecules released by injured tissue (such as HMGB1 (Bianchi, 2009; Bianchi and Manfredi, 2009)) which may be critically important under pathological conditions. TLR4 expression was also detected in motor neurons, consistent with reports of their critical role in regulating neuronal activity (for reviews see Crack and Bray, 2007; Mallard et al., 2009). Neuronal TLR4 expression has been recently shown in both experimental and human epileptic tissue, supporting a critical role for the HMGB1-TLR4 pathway in the regulation of neuronal excitability and neurotoxicity by enhancing neuronal calcium influx through NMDA receptor.
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(Maroso et al., 2010). Thus, activation of neuronal TLR4 could play a role in the progressive degeneration of motor neurons in ALS.

**Cellular distribution of RAGE in ALS spinal cord**

In the ALS spinal cord, RAGE protein was consistently expressed in activated astrocytes and microglial cells, as well as in the residual motor neurons. Surprisingly, however, no significant changes were observed in RAGE mRNA expression in total homogenates of ALS specimens compared to control spinal cord. This observation may reflect the loss of neurons expressing RAGE in ALS specimens and hence a lack of increase in overall RAGE levels in RT-PCR. RAGE binds different molecules, including HMGB1 and members of the S100 protein family (Sims et al., 2010). Interestingly, up-regulation of RAGE expression in reactive astrocytes and microglial cells has been reported in patients with Alzheimer’s and Huntington’s disease (Sasaki et al., 2001; Ma and Nicholson, 2004; Lue et al., 2005). Targeting RAGE has been suggested as potential therapeutic strategy to attenuate neurodegeneration (Ramasamy et al., 2005; Sturchler et al., 2008).

**Figure 7:** Evaluation of TLR2, TLR4, RAGE and HMGB1 glial immunoreactivity (IR) in control and ALS spinal cord. Plots showing the distribution of TLR2 (A) and TLR4 (B), RAGE (C) and HMGB1 (D) glial IR in controls and ALS spinal cord. The IR score represents the total score, which was taken as the product of the intensity score and the frequency score (for details see Experimental procedures section); panel (D) shows the distribution of the total score for HMGB1 in glial cells with both cytoplasmic and nuclear IR. ALS-st, patients with rapid disease progression and short-term survival <18 mon; ALS-lt, patients with slow disease progression and long-term survival >48 mon.
Cellular distribution of HMGB1 in ALS spinal cord

HMGB1 a predominantly nuclear protein can act as a potent proinflammatory cytokine-like mediator, thus contributing to amplification of the inflammatory response (Bianchi and Manfredi, 2007; Bianchi, 2009; Hreggvidsdottir et al., 2009). In histologically normal spinal cord HMGB1 was mainly expressed in nuclei of glial cells as reported in human control hippocampus (Maroso et al., 2010). In ALS spinal cord, HMGB1 IR showed variable nuclear/cytoplasmic expression in motor neurons although both nuclear and cytoplasmic expression was observed in reactive astrocytes and activated microglia. A cytoplasmic translocation has been recently reported in both microglia and astrocytes in experimental models of seizures and in tissue from epileptic patients with hippocampal sclerosis (Maroso et al., 2010).

In the spinal cord of SOD1G93A transgenic ALS mice a reduction of HMGB1 IR has been observed in degenerating neurons during the progression of the disease, whereas reactive glial cells displayed HMGB1 IR in the nucleus, but not in the cytosol (Lo Coco et al., 2007). In our study on human tissue (end stage of disease), we could not detect a significant change in the number of motor neurons showing nuclear and/or cytoplasmic staining in ALS spinal cord. However, we observed a cytoplasmic translocation of HMGB1 IR in activated microglia and astrocytes, supporting the role of glial cells as major source of extracellular HMGB1 in ALS patients with both rapid and slow disease progression. Relocation of the nuclear protein to the cytoplasm has been shown to be induced in rat (Hayakawa et al., 2010) and human Zurolo et al., 2011 cultured astrocytes by an inflammatory mediator like IL-1β. In addition, both microglia and astrocytes have been also shown to respond to HMGB1 stimulation with induction of several inflammatory mediators (Kim et al., 2006; Pedrazzi et al., 2007; Andersson et al., 2008). Thus, our observations suggest that both astrocytes and microglia may provide a positive feedback loop that amplifies the inflammatory response in human ALS. In addition, HMGB1 secreted by activated glia may contribute to selective progressive death of motor neurons, via a signaling pathway involving the TLR4 (Maroso et al., 2010). We did not observe differences between patients with different disease duration. However, we acknowledge that our sample size is relatively small, thus a large cohort of cases is required to evaluate any possible correlation with disease severity and duration.

Conclusion

In conclusion, our findings suggest activation of the TLR/ RAGE signaling pathways in human ALS. The potential link between TLR/RAGE signaling, progression of inflammation and motor neuron degeneration may suggest new therapeutic strategies, targeting inflammation, to be further explored in ALS.

Reference List


Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue


Part II: chapter 8


