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RESEARCH ARTICLE

Serum Nogo-A levels are not elevated in amyotrophic lateral sclerosis patients

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Abstract
Improved biomarkers would facilitate the diagnosis and treatment of amyotrophic lateral sclerosis (ALS). Muscle content of the neuritic outgrowth inhibitor Nogo-A is increased in patients with ALS and other denervating conditions. Seeking a less invasive diagnostic method, we sought to determine whether or not Nogo increases in the serum of ALS patients. We developed a dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) protocol to screen serum samples from 172 ALS patients and 172 healthy controls for Nogo-A immunoreactivity. Unexpectedly, there was a trend toward decreased levels of serum Nogo-A in ALS. Mean serum Nogo-A level in ALS patients was 0.71 nM (95% confidence interval (CI) 0.42–1.00), as opposed to 1.15 nM (95% CI 0.72–1.59) in healthy controls. A significantly larger percentage of healthy control sera (11.0% vs 4.7%) displayed markedly elevated levels of Nogo-A. Additional study is required to determine the factors that lead to elevated Nogo-A levels in a subset of both ALS patients and healthy controls.

Keywords: *ALS; Nogo-A; biomarker; DELFIA; ELISA*

Introduction

Diagnosis of the sporadic form of the fatal motor neuron degenerative disorder amyotrophic lateral sclerosis (ALS) depends on clinical criteria and electromyographic evaluation for definitive confirmation. Despite progress in the field, radiographic tests such as diffusion tensor tractography and serum biomarkers such as angiogenin have not yet achieved widespread utility in this clinically heterogeneous disorder (Cronin et al. 2006, Iwata et al. 2008). Quantitative non-invasive tests would improve not only the diagnosis of ALS but also the tracking of disease progression over time (Pasinetti et al. 2006).

Mounting evidence suggests a link between the Nogo-A protein and ALS. Nogo-A is best known for its role as a myelin-associated inhibitor of nerve sprouting and regeneration (Liu et al. 2006). However, Nogo isoforms are also expressed within skeletal muscle and neurons, where their function is not clear (Oertle & Schwab 2003, Yang & Strittmatter 2007). In a mouse model of ALS carrying a glycine to arginine mutation at residue 86 of superoxide dismutase 1 (SOD1), Nogo expression was found to increase in lumbar spinal cord and gastrocnemius muscle extracts (Dupuis et al. 2002). Similar results have been obtained in the G93A (glycine to alanine at residue 93 of SOD1) mouse model of ALS (Yang et al. unpublished). Furthermore, Nogo-A protein levels increase in muscle biopsy tissue from human ALS patients correlating with disease severity (Jokic et al. 2005). Levels of muscle Nogo-A expression may also predict progression to definite ALS in patients initially presenting with lower motor neuron weakness of unclear origin (Pradat et al. 2007). However, the specificity of muscle Nogo-A as a biomarker for ALS relative to other denervating neuromuscular diseases remains controversial (Wojcik et al. 2006, Teng & Tang 2008, Tagerud et al. 2007, Magnusson et al. 2003).

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The added time, expense and expertise required for muscle biopsies in ALS and certain other neuromuscular patients may outweigh the benefits, especially if one were to seek multiple biopsies over time. Therefore, we sought to establish a sensitive assay for detection of full-length Nogo-A or fragments released into serum. We hypothesized that elevated muscle Nogo-A expression in ALS patients would lead to elevated serum Nogo-A levels, either through release of cellular contents, or through shedding from cellular surfaces. We developed a sandwich antibody assay to analyse sera from 172 ALS patients and 172 healthy controls for Nogo-A levels.

Materials and methods

Participant samples

De-identified sera were obtained from participants with ALS and healthy controls as part of an IRB-approved ALS sample repository study. Except for one sample from a patient with a diagnosis of primary lateral sclerosis, other samples were from patients with probable or definite ALS according to El Escorial criteria. Of the ALS cases 163 were sporadic and nine were familial. All participants provided written informed consent. Purified Nogo-A standards were purified from HEK cells transiently transfected with a plasmid encoding residues 1–1040 of human Nogo-A with carboxyl-terminal myc and histidine tags (Fournier et al. 2001).

Serum Nogo-A immunoassay

Dissociation-enhanced lanthanide fluorescence immunoassays (DELFIA) rely on detection of the fluorescent lanthanide Europium (Allicotti et al. 2003). To detect human Nogo-A with maximum sensitivity, we used a 'sandwich' DELFIA assay. Briefly, 96-well assay plates (Nunc, Rochester, NY, USA) were coated with 333 ng of gelatin-free goat anti-Nogo-A (raised against a peptide containing amino acids 1–18 of human Nogo-A; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in 50 mM sodium bicarbonate buffer (pH 9.2) at room temperature for 25–27 h. Following a brief wash with Tris-buffered saline (TBS), plates were blocked with saline containing 0.5% BSA and 0.1% Tween-20 at 4°C for overnight or longer. Serum samples were then added at a volume of 20 µl diluted with 30 µl of blocking buffer, and incubated for 2 h at room temperature. All samples were assayed in triplicate plates, with a subset of samples repeatedly assayed to ensure experiment-to-experiment reproducibility. Plates were washed extensively with TBS containing 0.1% Tween-20 (TBST) after sample binding and all subsequent steps. Affinity-purified rabbit anti-Nogo-A (raised against a peptide encoding amino acids 623–640 of human Nogo-A) (Wang et al. 2002) was then added at a dilution of 1:1000 in blocking buffer and incubated for 1–2 h at room temperature. Biotinylated antirabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) was then added at a dilution of 1:1000 for 1 h at room temperature. Streptavidin-conjugated Europium (Perkin-Elmer 1244-360, Waltham, MA, USA) was then added at a dilution of 1:500 for 1 h at room temperature. For detection of bound Europium following the last set of washes, an acidic enhancer solution (Perkin-Elmer 1244-105) was added to dissociate the Europium from streptavidin. Plates were incubated with enhancer solution for 5 min at 25°C with shaking in a Victor$_3$-V spectrophotometer (Perkin-Elmer). Fluorescence was then detected by excitation at 340 nm and emission at 615 nm.

After optimization of individual reagent concentrations, the Nogo-A DELFIA could detect as little as 0.18 nM purified Nogo-A mixed with albumin solution or with control human serum (signal to noise ratio >2). With higher amounts of Nogo-A, the signal to noise ratio is greater than 100, where noise is defined as the output of a reaction missing any one component. Serial dilutions of Nogo-A standards display linear changes in fluorescence ($r^2 = 0.9989$). Intra- and interexperiment variability is negligible.

Statistical analysis

Counts of Europium fluorescence were converted into absolute serum Nogo-A concentrations using Microsoft Excel by calculating the slope of serial dilutions of purified Nogo-A standards diluted in control human serum. Error bars represent standard deviations of triplicate plates. Further statistical analysis (95% confidence intervals (CI), Mann–Whitney *U* test for non-normal distributions) was performed using SPSS 16.

Results

Serum Nogo-A levels are not increased in ALS patients

Nogo-A DELFIA was performed in triplicate on all control and ALS sera by an investigator unaware of diagnosis (N.Y.H.). Results are depicted in Figure 1. Contrary to expectations, ALS sera demonstrated a tendency toward lower Nogo-A values than healthy control sera. Mean serum Nogo-A values were 0.71 ± 0.15 nM for ALS patients (95% CI 0.42–1.00) and 1.15 ± 0.22 nM for healthy controls (95% CI 0.72–1.59). Although most sera demonstrated low-level Nogo-A reactivity (75.6% of all samples below 0.5 nM), a subset of samples in both groups showed markedly higher Nogo-A concentrations. Whereas 19 control sera (11.0%) contained

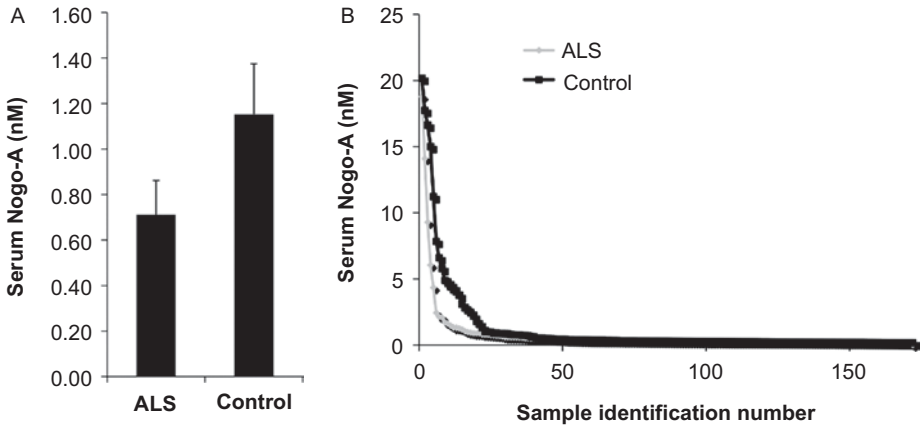

Figure 1. Serum Nogo-A levels are not elevated in amyotrophic lateral sclerosis (ALS). One hundred and seventy two samples each of healthy control and ALS patient sera were tested for Nogo-A immunoreactivity using a sandwich DELFIA assay (see text). (A) Overall means for each group (healthy controls 1.15 ± 0.22 nM, 95% confidence interval (CI) 0.72–1.59; ALS patients 0.71 ± 0.15 nM, 95% CI 0.42–1.00). (B) Breakdown of individual sample values revealed a small subset of both groups with much higher serum Nogo-A concentration (control 11.0 %, ALS 4.7%). The distribution of serum Nogo-A values between ALS patients and healthy controls was found to be significantly different using the Mann–Whitney *U* test for non-normal distributions ($p = 0.03$).

Nogo-A at levels greater than 2 nM, only eight ALS sera (4.7%) had levels greater than 2 nM. The distribution of serum Nogo-A values between ALS patients and healthy controls was found to be significantly different using the Mann–Whitney *U* test for non-normal distributions ($p = 0.03$).

Discussion

We hypothesized increased Nogo-A concentration in ALS patient serum based on findings by another group of increased Nogo-A expression in ALS patient muscle (Dupuis et al. 2002, Jokic et al. 2005). Although Nogo-A is a transmembrane protein that is not normally secreted, we reasoned that atrophic changes often associated with ALS could result in release of Nogo-A or fragments of Nogo-A into the bloodstream, in a similar fashion to the release of creatine kinase from dying muscle (Achari & Anderson 1974). A precedent for detectable Nogo isoforms in human serum exists (Rodriguez-Feo et al. 2007). Moreover, a precedent exists for the use of decreased rather than increased concentrations of specific CSF peptides such as fragments of cystatin 3 and VGF as biomarkers for ALS (Pasinetti et al. 2006).

Although the distribution of values for serum Nogo-A was significantly lower in ALS patients than healthy controls in our study, the mean values were not significantly different. Several possible explanations could underlie these findings. Degenerating muscle may release carboxyl-terminal Nogo-A fragments that would not be detected by our assay, which depends on the presence of Nogo-A residues 1–18 through 623–640 in serum. Alternatively, muscle atrophy may have been so advanced by the time of blood collection in ALS patients that serum Nogo-A levels may have already decreased after potentially increasing earlier during the disease course. A post-hoc analysis demonstrated no correlation between serum Nogo-A values and duration of disease symptoms (not shown). A study to follow Nogo-A levels over time within individual ALS patients could shed light on this possibility – our serum-based assay makes this approach much more feasible than performing multiple muscle biopsies.

Interestingly, a subset of subjects showed highly elevated Nogo-A serum values. This occurred more frequently in the healthy control group than the ALS group. We performed a separate experiment to determine whether these high Nogo-A values could be explained by inadvertent haemolysis of our serum samples. When Nogo-A levels were compared in whole venous blood obtained from five healthy volunteers separated into serum, plasma and cell pellet homogenate, values were 84% higher for the cell homogenate, but still well below the 2 nM cut-off used to delineate 'highly elevated' serum Nogo-A values (not shown). Thus, even if there were varying levels of haemolysis among our 344 serum samples, this would not cause a sufficient rise in Nogo-A levels to explain the subset of samples with values higher than 2 nM. We do not have matching muscle biopsy samples to determine whether elevated serum Nogo-A correlates with elevated muscle Nogo-A in healthy controls or ALS patients. However, these findings add to evidence that increased Nogo-A expression may not be specific for ALS (Wojcik et al. 2006, Teng & Tang 2008, Magnusson et al. 2003, Tagerud et al. 2007). It will be of great interest to determine other potential causes of elevated serum Nogo-A, and whether this elevation has any phenotypic effect.

In summary, unlike muscle Nogo-A, concentrations of serum Nogo-A do not increase in ALS.

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***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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