Journal of Neuroimmunology 220 (2010) 114–119

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

A highly sensitive electrochemiluminescence immunoassay for the neurofilament heavy chain protein

Jens Kuhle [a,*], Axel Regeniter [b], David Leppert [a], Matthias Mehling [a], Ludwig Kappos [a], Raija L.P. Lindberg [a,1], Axel Petzold [c,1]

[a] Clinical Neuroimmunology, Neurology and Department of Biomedicine, University Hospital Basel, Switzerland
[b] Laboratory Medicine, University Hospital Basel, Switzerland
[c] Department of Neuroimmunology, UCL Institute of Neurology, Queen Square, London, UK

ARTICLE INFO

Article history:
Received 31 October 2009
Received in revised form 31 December 2009
Accepted 5 January 2010

Keywords:
Neurofilament heavy chain (NfH)
Electrochemiluminescence (ECL)
Multiple sclerosis
Amyotrophic lateral sclerosis
Guillain–Barré syndrome
Mild cognitive impairment/Alzheimer's disease

ABSTRACT

Background: The loss of neurological function is closely related to axonal damage. Neurofilament subunits are concentrated in neurons and axons and have emerged as promising biomarkers for neurodegeneration. Electrochemiluminescence (ECL) based assays are known to be of superior sensitivity and require less sample volume than conventional ELISAs.
Methods: We developed an ECL based solid-phase sandwich immunoassay to measure the neurofilament heavy chain protein (NfH^{SMI35}) in CSF. We employed commercially available antibodies as previously used in a conventional ELISA (Petzold et al., 2003; Petzold and Shaw, 2007). The optimised and validated assay was applied in a reference cohort and defined patient groups.
Results: Analytical sensitivity (background plus three SD) of our assay was 2.4 pg/ml. The mean intra-assay coefficient of variation (CV) was 4.8% and the inter-assay CV 8.4%. All measured control and patient samples produced signals well above background. Patients with multiple sclerosis (MS) (median 46.2 pg/ml, $n=95$), amyotrophic lateral sclerosis (ALS) (160.1 pg/ml, $n=50$), mild cognitive impairment/Alzheimer's disease (MCI/AD) (65.6 pg/ml, $n=20$), Guillain–Barre syndrome (GBS) (91.0 pg/ml, $n=20$) or subarachnoid hemorrhage (SAH) (345.0 pg/ml, $n=20$) had higher CSF NfH^{SMI35} values than the reference cohort (27.1 pg/ml, $n=73$, $p<0.0001$ for each comparison).
Conclusion: The new ECL based assay for NfH^{SMI35} in CSF is superior in terms of sensitivity, precision and accuracy to previously published methods (Petzold et al., 2003; Shaw et al., 2005; Teunissen et al., 2009). The improved performance and small sample volume requirement qualify this method in experimental settings and clinical trials designed to perform a number of tests on limited amounts of material.

1. Introduction

Highly sensitive methods for detecting soluble biomarkers for neuro-axonal damage are needed in neurodegenerative diseases. The three main applications for such biomarkers in clinical settings are to improve individual 1) diagnostic accuracy and, 2) prediction of disease course and treatment response and 3) to serve as cross-sectional surrogate endpoint in observational or therapeutic trials (McShane et al., 2006). Neurofilaments (Nf), proteins specifically expressed in neurons and axons, have emerged as promising biomarkers for neurodegeneration experimentally and clinically in a range of neurological disorders (Petzold et al., 2004a,b; Rosengren et al., 1996; Teunissen et al., 2009; Deisenhammer et al., 2009).

Nf are among the most abundant proteins of the nervous system, composed of four subunits: the triplet of the NF light (NfL), NF medium (NfM) and heavy chain (NfH) and α-internexin in the CNS or peripherin in the PNS (Lee and Cleveland, 1996; Herrmann and Aebi, 2000). NfH is the most extensively phosphorylated protein of the human brain with regulatory influences on cell structure homeostasis and axonal transport and is released into the CSF following axonal damage, predominantly in acute neurological diseases (de Waegh et al., 1992; Roy et al., 2000; Petzold, 2005; Teunissen et al., 2005; Deisenhammer et al., 2009).

Several in-house NfH-assay protocols have been developed in recent years (Petzold et al., 2003; Shaw et al., 2005; Teunissen et al., 2009) and two commercial NfH ELISA kits are available (EnCor Biotechnology Inc., Gainesville, FL, licensed by Millipore, Billerica, MA; BioVendor, Modrice, Czech Republic).

* Corresponding author. Clinical Neuroimmunology, Neurology and Department of Biomedicine, University Hospital Basel, Petersgraben 4, CH-4031 Basel, Switzerland. Tel.: +41 61 265 25 25; fax: +41 61 265 4100.
E-mail address: kuhlej@uhbs.ch (J. Kuhle).
[1] These authors contributed equally.

0165-5728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2010.01.004

Electrochemiluminescence (ECL) based assays are known to be highly sensitive, exhibit a broad dynamic range and require small sample volume; ECL technology has demonstrated the ability to quantitate levels of nucleic acids, recombinant proteins and bacterial and viral components in the sub-picogram range with increased precision compared to conventional ELISA (Motmans et al., 1996; Yu and Bruno, 1996; Grimshaw et al., 1997; Kijek et al., 2000; Guglielmo-Viret et al., 2005, 2007).

We aimed to develop and validate an ECL solid-phase sandwich immunoassay to measure the soluble fraction of NfH^{SMI35} in CSF employing the same commercially available antibodies as previously used in a conventional ELISA (Petzold et al., 2003; Petzold and Shaw, 2007). Adhering to a previously proposed nomenclature the soluble fraction of NfH measured is indicated with the capture antibody in the superscript (NfH^{SMI35}) (Petzold et al., 2003). We concentrated on NfH^{SMI35} because of its functional importance in axonal pathology (Hisanaga et al., 1993; Elder et al., 1998; Gou et al., 1998; Elder et al., 1999; Jacomy et al., 1999; Roy et al., 2000) and its relative resistance to proteolytic cleavage, a prerequisite for reliable measurement in body fluids (Schlaepfer et al., 1985; Goldstein et al., 1987; Pant, 1988).

We describe a new ECL based assay which is superior in terms of sensitivity, precision and accuracy to previously published methods (Petzold et al., 2003; Shaw et al., 2005; Teunissen et al., 2009). Moreover, because of the smaller sample volume requirement this method will allow to perform accurate measurements in experimental and clinical settings where available sample volume is a limiting factor with conventional ELISA methods.

2. Materials and methods

2.1. Antibodies

The following antibodies were used: capture monoclonal antibody SMI 35 (Covance, Emeryville, CA), the secondary (detector) polyclonal rabbit anti-NfH^{SMI35} antibody (Sigma-Aldrich, Saint Louis, MO), and the indicator polyclonal Sulfo-TAG labeled goat anti-rabbit antibody (ruthenylated) (MSD, Gaithersburg, MD).

2.2. Chemicals

Barbitone, bovine serum albumin (BSA), ethylenediaminetetraacetic disodium salt (EDTA), NaCl, phosphate buffered saline (PBS), tris base and Tween 20 were of analytical grade (Sigma-Aldrich, Saint Louis, MO).

2.3. Standards

Bovine NfH was obtained from USBiological (United States Biological, Swampscott, MA). Standards were diluted in tris buffered saline (TBS) containing 1% BSA, 0.1% Tween 20 and 0.06 mM EDTA, pH 7.5 and ranged from 0 to 2500 pg/ml. The standards were stored at −20 °C.

2.4. CSF samples

CSF samples were obtained by lumbar puncture as part of the routine diagnostic procedure, except the patients ($n=20$) with a subarachnoid hemorrhage where the CSF was taken for routine infectious screening two days after placement of an extraventricular drainage (EVD). Within 2 h the CSF samples were centrifuged at 400 *g* for 10 min at room temperature and aliquoted in polypropylene tubes and stored at −80 °C. Pooled CSF was obtained by taking an aliquot from routine samples and stored at −80 °C until further analysis. Samples were collected in the Department of Neurology, University Hospital Basel in the course of routine diagnostic measures as indicated by the treating physicians and after patient informed consent.

The control group ($n=73$) consisted of patients who, based on extensive diagnostic evaluation had no objective clinical or paraclinical (cranial MRI, CSF analysis, EEG and additional tests as indicated) signs of a neurological disease. These patients suffered from tension type headache ($n=17$), lower back pain ($n=5$), psychiatric disorders ($n=30$) or miscellaneous diseases for which no neurological explanation could be found ($n=21$).

In addition to the controls 95 patients with MS, 50 patients with amyotrophic lateral sclerosis (ALS), patients with a mild cognitive impairment/Alzheimer's disease (MCI/AD), a Guillain–Barré syndrome (GBS) or subarachnoid hemorrhage (SAH) ($n=20$ each) were included. The patient data was blinded during the analytical procedure.

2.5. Analytical procedure

The 96-well plates (Multi-Array® plates, Meso Scale Discovery, Gaithersburg, MD) include integrated screen-printed carbon ink electrodes on the bottom of the wells. Coating was done overnight with 25 µl of capture antibody diluted 1/2500 in PBS (pH 7.4). All following incubation steps were done with vigorous shaking (800 rpm) and were preceded by three wash steps with 200 µl of TBS containing 0.1% Tween 20 (pH 7.5) per well. Unspecific binding sites were blocked with 25 µl of TBS containing 3% BSA per well for 1 h. After washing, 25 µl of TBS containing 1% BSA, 0.1% Tween 20 and 50 mM Barbitone was added as sample diluent to each well. 25 µl of standard, control or CSF sample was then added in duplicate and the plate incubated at room temperature (RT) for 1 h. After washing, 25 µl of the secondary antibody diluted 1/2000 in TBS containing 1% BSA, 0.1% Tween 20 and 50 mM Barbitone was added to each well and the plate was incubated for 2 h at RT. After washing Sulfo-TAG labeled goat anti-rabbit antibody diluted 1/2000 in TBS containing 1% BSA and 0.1% Tween 20 was added and incubated for 1 h at RT. Following a final wash, 150 µl of ECL read buffer (MSD) diluted 1:2 with distilled water was added and the ECL signal, detected by photodetectors, was measured using the MSD Sector Imager 2400 plate reader. A four-parameter weighted logistic fit curve was generated, sample concentrations extrapolated and analysed using the Discovery Workbench 3.0 software (MSD).

2.6. Statistical evaluation

Continuous variables are presented as median with interquartile range (IQR), categorical variables as numbers and percentages. Comparisons between more than two groups were done by Kruskal–Wallis analysis of variance on ranks and general linear models. Two group comparisons were performed by Mann–Whitney *U* test. CSF levels of NfH^{SMI35} were log-transformed to achieve a normal distribution. To identify confounding factors such as age or gender, linear regression analysis was assessed by univariate and multivariate analysis. Multiple correlations were corrected using the Bonferroni method. The cut-off (upper reference range of normal) for CSF NfH^{SMI35} was defined as the highest value observed in the control group. Proportions of patients above and below this cut-off were compared with the Chi-Square test. The linear relationship between continuous variables was evaluated using the Spearman correlation coefficient. Linear regression analysis was performed using the least-squares method. All statistical analyses and graphs were prepared using SPSS (Version 15.0 SPSS, Chicago, IL), SAS software (version 9.2, SAS Institute, Cary, NC) and Graph Pad Prism 5.02 for Windows (GraphPad Software, San Diego, CA). A two-sided $p<0.05$ was considered as statistically significant.

3. Results

3.1. Reproducibility of the standard curve

Standards were prepared as four-fold serial dilutions ranging from 0 to 2500 pg/ml in sample diluent. Fig. 1 shows the average of 20 consecutive normalized standard curves in the range of 0–2500 pg/ml by expressing the counts obtained for each calibrant as the percentage