Sez6 levels are elevated in cerebrospinal fluid of patients with inflammatory pain–associated conditions

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Abstract

Introduction: Seizure-related protein 6 (Sez6) contributes to chronic pain development as *sez6* knockout mice show attenuated pain behaviours after peripheral nerve injury, compared with control mice. The type I transmembrane isoform of Sez6 is cleaved by the β-amyloid precursor protein cleavage enzyme 1 (BACE1), resulting in Sez6 extracellular domain shedding from the neuron surface.

Objectives: To determine whether this BACE1-shed form of Sez6 can be detected in the cerebrospinal fluid (CSF) and whether Sez6 levels in the CSF are altered in neuropathic pain or chronic inflammatory pain (IP).

Methods: We analysed the CSF samples collected during surgery from patients with chronic neuropathic pain (n = 8) or IP (n = 33), comparing them to the CSF samples from patients with suspected subarachnoid haemorrhage that was subsequently excluded (nonsurgical group, n = 5). Western blots were used to determine the relative Sez6 levels in the CSF from the different patient and nonsurgical comparison groups.

Results: The results show that BACE1-shed Sez6 can be readily detected in the CSF by Western blot and that the levels of Sez6 are significantly higher in the IP group than in the nonsurgical comparison group.

Conclusion: The association between elevated Sez6 levels in the CSF and IP is further evidence for persistent alterations in central nervous system activity in chronic IP conditions.

Keywords: Seizure-related protein 6, CSF, Inflammatory pain, Chronic pain, BACE1

1. Introduction

Neuropathic and other forms of chronic pain affect 7% and 13% of the adult population, respectively.[17] Neuropathic pain (NP) arises from a nervous system injury that results in abnormal or spontaneous firing of afferent nociceptive and mechanosensory neurons, a lowered activation threshold of second-order neurons in the spinal cord dorsal horn, and hypersensitisation to stimuli such as heat and touch.[2,4]

We showed previously that Seizure-related protein 6 (Sez6) is important for the development of neuronal dendrites and synapses.[5,15] New findings from our laboratory, using a mixed pain model (chronic constriction injury) in mice, have revealed that Sez6 contributes to the development of chronic hyperalgesia and neuroinflammation after nerve injury. In neurons, Sez6 is almost exclusively cleaved by β-amyloid precursor protein cleaving enzyme 1, also known as β-secretase 1 or BACE1.[12,25] After cleavage of the transmembrane isoform of Sez6 by BACE1, the shed extracellular domain of the protein is released into the cerebrospinal fluid (CSF).[20]

Because the development of NP and chronic inflammatory pain (IP) involves increased excitatory drive into the spinal cord and into the brain through ascending pathways,[7,16,26,27] we aimed to test the hypothesis that higher levels of shed Sez6 in the CSF are associated with clinically diagnosed NP or IP. Specifically, this study addressed whether levels of Sez6 in the CSF are significantly changed in surgical patients with (1) chronic NP or (2) chronic IP, compared with a nonsurgical comparison group.
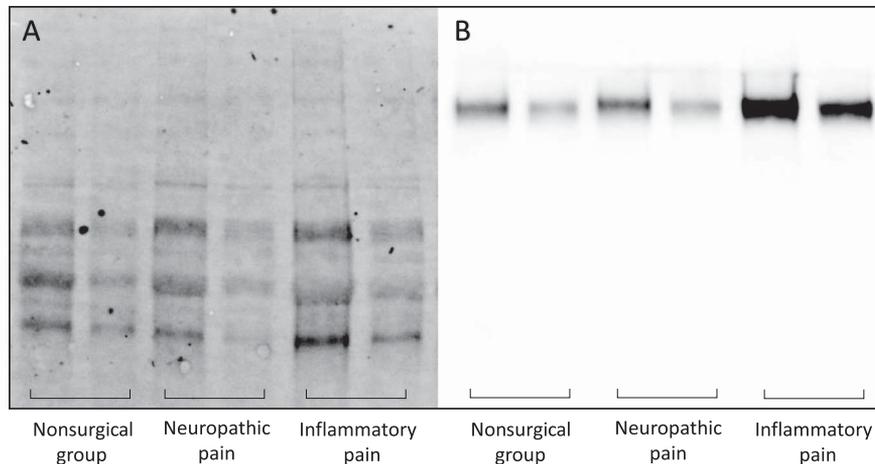
Figure 1. Relative Sez6 levels in the CSF by condition. Example of (A) stain-free and (B) Chemi Hi-Resolution Western blots used for analysis of total protein and Sez6 protein levels, respectively. For each sample, 10 or 5 µL was loaded (left or right lanes of each pair, respectively). CSF, cerebrospinal fluid.

2. Materials and Methods

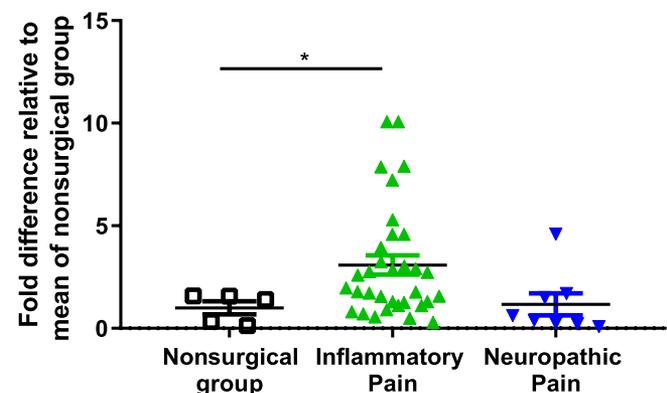### 2.1. Cerebrospinal fluid samples

Cerebrospinal fluid samples from patients undergoing surgery for painful conditions, categorised into NP or IP groups, were obtained from The Alfred Hospital, Melbourne, Victoria, Australia (Alfred Ethics Committee Project No: 247/13), with informed consent. Details of diagnosis and/or surgery are described in Appendix, Table 1 (available at http://links.lww.com/PR9/A41). Prescribed medications (see Appendix, Table 1, available at http://links.lww.com/PR9/A41) were also taken on the day of CSF collection. Pain severity and intensity scores on the modified Brief Pain Inventory (mBPI) scale were recorded. Cerebrospinal fluid was procured through lumbar puncture before any administration of anaesthetics or analgesics intrathecally, centrifuged to remove any contaminating blood cells, and stored at −80°C. Patient age and sex data are summarised in Appendix, Table 2 (available at http://links.lww.com/PR9/A41).

Cerebrospinal fluid samples in the "nonsurgical" comparison group were collected from patients presenting to the emergency department at the Royal Melbourne Hospital, Parkville, Victoria, Australia (RMH Ethics Committee Project ID: HREC 2012-050), with informed consent. Patients in this group presented with a history of sudden-onset headache and, as part of the diagnostic workup, received computed tomography brain scans (that showed no evidence for a subarachnoid haemorrhage) followed by a lumbar puncture, which definitively excluded subarachnoid haemorrhage in all patients in this group. The headaches in these individuals resolved and the cause was not further investigated but presumed to be migraine/tension headache. As indicated in the Appendix, one of these patients had taken Panadol before admission.

2.2. Protein assay and Western blot

Sample protein concentration was measured with the DC Protein Assay (Bio-Rad, Hercules, CA). For Western blots, CSF samples (100 µL) were prepared as follows: 100% trichloroacetic acid was added (10% vol/vol), the samples were then incubated at room temperature for 5 to 10 minutes, and centrifuged (4°C, 20,000g) for 5 minutes. Precipitated protein pellets were dissolved in 100 µL 1x Laemmli buffer, and the pH was adjusted to >pH 4.6 with ammonia vapour.

For each sample, 2 wells of a Mini-Protean 7.5% TGX precast polyacrylamide gel (Bio-Rad) were loaded (with 5 or 10 µL). Gels were transferred onto nitrocellulose membranes using the Trans-Blot Turbo semidry blotter (Bio-Rad). Membranes were blocked with skim milk powder (5% wt/vol) in 1x tris-buffered saline with 0.05% Tween-20 and incubated with a primary monoclonal antibody (rabbit anti-Sez6, 1/1000 in blocking solution) as described previously,[5] followed by a goat anti-rabbit HRP-conjugated secondary antibody (Upstate, ID: 12-348), diluted 1/10,000 in blocking solution. Sez6 bands were detected using enhanced chemiluminescence (Clarity ECL, Bio-Rad). The Molecular Image Chemidoc MP System and Image Lab software (Bio-Rad) were used to create a multichannel image. Total protein load was normalised from the Stain-Free blot image, and the specific Sez6 protein signal in each lane was measured from the Chemi Hi-Resolution image (exposure time 90 seconds). An average integrated density value for the Sez6 signal was calculated for each CSF sample. Fold differences were calculated relative to the normalization standard or nonsurgical comparison sample run on the same gel and then the relevant adjustment factor was applied to compare all values with the mean value of all the nonsurgical group

Figure 2. Cerebrospinal fluid Sez6 levels are significantly elevated in inflammatory, but not neuropathic, pain patients compared with samples from the nonsurgical comparison group. A significant increase in shed Sez6 levels in patients with inflammatory pain (n = 33, $P < 0.05$) was detected compared to samples from the nonsurgical comparison group (n = 5). No significant difference in patients with neuropathic pain (n = 8, $p > 0.05$) was observed compared to the control samples. CSF, cerebrospinal fluid.
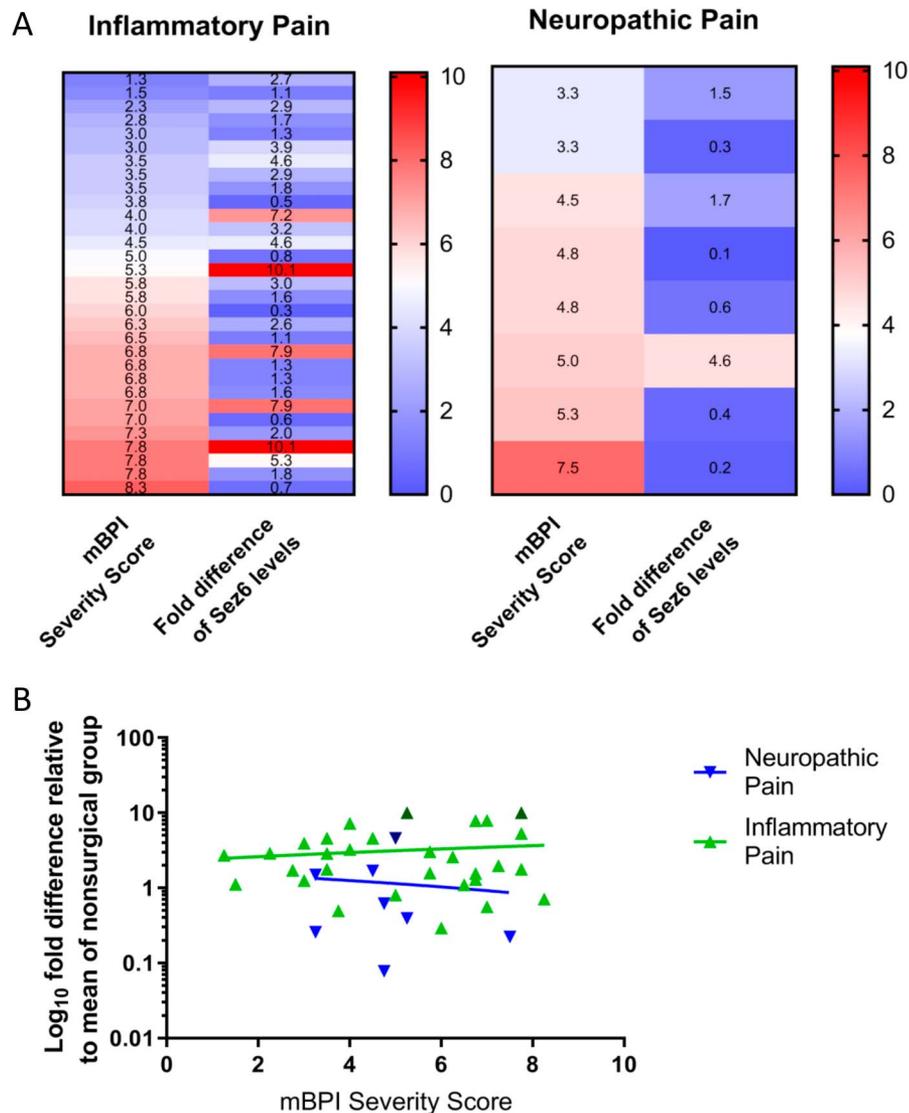
Figure 3. Relative CSF Sez6 levels are not correlated with pain severity scores. (A) Heat maps of modified Brief Pain Inventory (mBPI) pain severity scores and relative Sez6 levels. (B) Linear regression of mBPI pain severity score in patients with neuropathic (y = −0.1115x + 1.703) and inflammatory (y = 0.1118x + 2.484) pain. Dark triangles represent patient outliers (1.5 × interquartile range) for relative Sez6 levels. Pain severity scores were not significantly correlated with CSF Sez6 levels ($P > 0.05$). CSF, cerebrospinal fluid.

samples (see Appendix, Tables 3 and 4 for integrated density values and calculations, available at http://links.lww.com/PR9/A41). Fold-difference ratios were $\log_{10}$-transformed to normalize the distribution.

2.3. Statistical analysis

Statistical significance was tested using one-way analysis of variance on $\log_{10}$-transformed data, followed by the Dunnett multiple comparisons test. The strength of the correlation between patients' pain severity scores and relative Sez6 levels in the CSF was tested (Pearson correlation).

3. Results

3.1. Sez6 is elevated in the cerebrospinal fluid of patients with inflammatory pain

Sez6 was readily detected in the CSF by Western blot (**Fig. 1**), and relative Sez6 levels in each CSF sample are plotted in **Figure 2**.

Levels of shed Sez6 were significantly higher in the CSF of patients with IP relative to the mean of all control samples (mean ± SEM fold difference = 3.09 ± 0.47 (IP), 1.00 ± 0.32 (controls); $P = 0.038$, n = 33 (IP), n = 5 (controls); **Fig. 2**). No significant difference in shed Sez6 levels in the CSF of patients with NP was observed, relative to controls (mean ± SEM fold difference = 1.17 ± 0.53 (NP), 1.00 ± 0.32 (controls); $P > 0.05$, n = 8 (NP), n = 5 (controls); **Fig. 2**).

3.2. No correlation between Sez6 levels and modified Brief Pain Inventory pain severity scores

Tukey boxplots of the Sez6 levels in the CSF from IP and NP groups revealed 2 outliers and 1 outlier, respectively (Appendix, Figure 1, available at http://links.lww.com/PR9/A41). All outliers had an mBPI pain severity score of 5.0 or higher (on a scale of 0–10). Heat maps and linear regression plots of mBPI scores against relative CSF Sez6 levels are shown in **Figure 3**. Severity scores were not correlated with Sez6 CSF levels in patients with

IP (Pearson $r = 0.13$, $R^2 = 0.0169$; $P > 0.05$) or NP ($r = -0.10$, $R^2 = 0.010$; $P > 0.05$).

4. Discussion

Patients with inflammatory, but not neuropathic, pain showed significantly elevated levels of shed Sez6 in the CSF compared with the control patients. In neither group were the CSF levels of Sez6 significantly correlated with reported pain scores. The lack of a strong correlation between various measures of inflammation and subjective measures of pain intensity has been previously reported[19] and is likely attributable to the multifactorial nature of pain.[1]

Osteoarthritis (the most common diagnosis of the patients with IP) involves release of inflammatory mediators[11] that are capable of sensitising peripheral nociceptors,[3] resulting in a lower activation threshold and increased firing of centrally projecting afferent axons in the spinal cord, even with normally innocuous stimuli.[10] Increased excitatory drive and local neuroinflammation, in turn, lead to central nervous system sensitisation,[9,21] including exaggerated and persistent synaptic long-term potentiation.[8] The known roles for Sez6 in the development and maintenance of excitatory synapses,[18] and the upregulation of Sez6 mRNA levels during long-term potentiation induction,[6] suggest that Sez6 may be involved in the activity-dependent chronification of pain and might explain the observed association between elevated Sez6 levels in the CSF and IP conditions.

Sez6 levels in IP samples seem to be segregating into 2 clusters. No obvious commonalities could be identified amongst patients with the highest Sez6 levels, although clearer patterns may emerge if the sample size were increased and/or serial samples were available. If medically indicated, analysing serial samples from individual patients would be preferable to single sample analysis, provided the protocol for repeated CSF collection was standardised. An important consideration, particularly for interpretation of the results presented here, is that levels of Aβ, itself a product of BACE1 activity, are known to vary diurnally as well as increasing with draw frequency.[13]

Medical biomarkers are important tools for identifying susceptibility to disease, predicting treatment success, and facilitating objective diagnoses.[24] A quantitative proteomics study indicated that Sez6 levels in the CSF are elevated in myalgic encephalomyelitis/chronic fatigue syndrome[23] and Sez6 is also implicated in psychiatric disorders, forming part of a CSF biomarker signature for schizophrenia, bipolar disorder, and major depressive disorder.[14] Although all 3 Sez6 family protein members are found in the CSF, only one (Sez6L2) has been identified in blood plasma.[22] With the enhanced sensitivity (compared with Western blot) of ELISA-based assays, currently under development for shed Sez6 proteins, detection of Sez6 in serum may soon become feasible.

Because Sez6 is almost exclusively cleaved by BACE1,[25] levels of shed Sez6 in the CSF can be used as a direct indicator of BACE1 activity. It will be important to determine whether Sez6 shedding contributes to IP and whether BACE1 inhibitors (currently in clinical trials for the treatment of Alzheimer disease) might be useful for treating conditions associated with elevated CSF Sez6 levels, such as chronic IP.

Disclosures

The authors have no conflict of interest to declare.

Acknowledgements

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PR9/A41.

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