HMGB1 as a predictive biomarker for drug-resistant epilepsy: a proof-of-concept study

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Summary

Currently no sensitive and specific biomarkers exist to predict drug-resistant epilepsy. We determined whether blood levels of high mobility group box-1, a mediator of neuroinflammation implicated in drug-resistant epilepsies, identifies patients with drug-resistant seizures. Patients with drug-resistant epilepsy express significantly higher levels of blood HMGB1 than those with drug-responsive, well-controlled seizures and healthy controls. No correlation existed between blood HMGB1 levels and total pre-treatment seizure count or days since last seizure at new epilepsy diagnosis indicating that blood HMGB1 does not solely reflect ongoing seizures. HMGB1 distinguishes with high specificity and selectivity drug-resistant vs drug-responsive patients. This protein has therefore potential clinical utility to act as a biomarker for predicting response to therapy which should be addressed in prospective clinical studies.

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Introduction

Drug-resistant seizures affect about a third of people with epilepsy. The discovery of biomarkers that can predict patients at risk of developing drug-resistance is likely to be critical to the success of clinical trials of novel therapeutics for the treatment or prevention of drug-resistance.

Mounting evidence suggests that dysregulated neuroinflammation in brain contributes to continued seizures and disease progression in drug-resistant structural epilepsies, and may modulate seizure response to anti-seizure drugs (ASDs) [1].

High Mobility Group Box 1 (HMGB1) mediates sterile neuroinflammation evoked by epileptogenic injury and recurrent seizures [2]. HMGB1 increases in neurons, glia and endothelial cells of the blood brain barrier (BBB) in human drug-resistant epilepsy foci, and in corresponding animal models [2,3]. Blood levels of HMGB1 appear to mirror brain changes in animal models of acquired epilepsy [3]. Mice injected intracerebrally with HMGB1 develop more seizures in response to chemoconvulsants whereas mice injected with anti-HMGB1 drugs or anti-HMGB1 monoclonal antibody or lacking HMGB1-activated TLR4 are less susceptible to seizures and less prone to develop epilepsy [2,3]. Finally, the HMGB1-TLR4 axis contributes to the overexpression of Pgp, a BBB protein, which is induced in drug-resistant epilepsy foci and extrudes various ASDs from the brain in human [4,5] and rodent epilepsy [6,7]. Thus, the collective evidence suggests that HMGB1 is implicated in epilepsy and may have therapeutic utility as a mechanistic biomarker for drug-resistance. We sought to elucidate whether blood HMGB1 level identifies epilepsy patients with drug-resistant vs drug-sensitive

seizures. This information is essential for developing prospective clinical studies to test the predictivity value of HMGB1 for drug-resistance.

Methods

We examined HMGB1 levels in sera from 65 patients with drug-resistant epilepsy (37 women and 28 men, mean age 34.8 years, range 17-65 years, Suppl. Table 1) attending for continuous inpatient video-EEG monitoring, 20 patients with well-controlled epilepsy (seizure-free on ASD therapy for >6 months, Suppl. Table 2) included as drug-responsive epilepsy controls, and 74 healthy controls. In a separate pilot study, we examined HMGB1 in the peripheral blood of 26 patients with newly diagnosed epilepsy at baseline. Patients were taking mono (12.5%), dual (37%), triple (38%) or 4 or more therapies (12.5%). There was no statistically significant relationship with the number of drugs administered and the level of serum HMGB1.

The protocols for the study involved identification of pharmacoresistance at recruitment. This was defined as failure of adequate trials of two tolerated, appropriately chosen and used ASD schedules plus listing for inpatient video-EEG as part of pre-surgical work-up for potential epilepsy surgery.

All patients provided written informed consent. The study protocols for the chronic epilepsy cohort were approved by the local ethics and research committees of both sites, North-West UK REC-Haydock (10/H1010/55) and the ethics committee at Tampere University Hospital. The newly-diagnosed cohort was approved by the West Research Ethics Committee, North Glasgow University (Glasgow, United Kingdom) NHS Trust in August 2003 (ref: 03/74, 1).

Chronic epilepsy cohort. Serum samples were obtained at baseline admission to the unit (minimum of 12 hours post-seizure) and prior to withdrawal of ASDs. Continuous video-electroencephalography (EEG) monitoring was undertaken in all patients (60:5 symptomatic:idiopathic) at the Walton Centre NHS Foundation Trust (n=15, prospective collection 2010-2013) and Tampere University Hospital, Finland (n=50, prospective collection 2004-2007). Ictal scalp recordings were obtained using synchronous digital video and 24-channel standard bipolar EEG for electroclinical characterization of their seizures as part of the routine clinical evaluation for possible epilepsy surgery. Magnetic resonance imaging (MRI) examination was undertaken on a 1.5 Tesla machine (General Electric Signa HD, Milwaukee, WI, U.S.A.). The International League Against Epilepsy (ILAE) diagnostic criteria were used to classify seizures and epileptic syndromes. Patients with any other co-morbidities other than epilepsy were excluded from analysis.

Controls. Healthy volunteers (n=74) without history of seizures (39 women and 35 men, mean age 34.1 years, range 19-66 years). Epilepsy controls comprised twenty patients with established in parenthesis12:8 monotherapy:dual therapy) who had been seizure free for longer than 6 months (12

women and 8 men, mean age 33 years, range 18-59 years). Baseline blood samples were obtained at the start of scalp recording.

Newly diagnosed cohort. Serum samples were obtained at pre-treatment baseline from a randomized trial of ASD monotherapy in newly diagnosed epilepsy conducted at Glasgow Western Infirmary, Scotland. This cohort comprised 26 patients (14 male) with a mean age at diagnosis of 29 years (range 17–60). Of these 26 patients, 13 had an initial diagnosis of focal epilepsy, 1 had primary generalized epilepsy, and 12 were unclassified at the time of randomization.

HMGB1 measurement. Serum extracted from whole blood was collected and aliquoted, then stored at -70°C until assayed. Serum samples were assigned random, linked numerical identifiers by the investigator aliquoting the samples. Total HMGB1 level was determined by a commercially available ELISA (Shino-test Corp, Sagamihara, Japan) according to the manufacturer's guidelines as described previously.

Statistical analysis

Statistical analysis was performed in SPSS. HMGB1 was compared between subjects using one-way ANOVA followed by Dunn's test, the association between MRI abnormalities and HMGB1 by t-test, sub-group analysis of MRI abnormalities by one-way ANOVA and compared to the normal MRI group via pairwise t-test with Bonferroni correction. ANOVA was performed to examine relationship between epilepsy subtype and baseline HMGB1. The false discovery rate was calculated for each test owing to the multiple tests undertaken. Continuous clinical variables were tested for association with HMGB1 using the t-test and categorical variables using $\chi 2$ or Fisher's exact test.

Results

Chronic epilepsy cohort. Patients with drug-resistant epilepsy had higher levels of HMGB1 (8.70 ± 0.47 ng/ml, n=65) than both healthy controls (1.11 ± 0.07 ng/ml, $p<0.01$, n= 74) and patients with drug-responsive epilepsy (1.25 ± 0.15 ng/ml, $p<0.01$; n=20, **Fig. 1a**). Drug-resistant patients with an abnormal brain MRI had significantly higher HMGB1 levels (mean ± s.e.m., 9.8 ± 0.7 ng/ml, n=35) than those without imaging abnormalities (7.4 ± 0.5 ng/ml, n=30, $p<0.01$; **Fig. 1b**). Sub-group analysis of the abnormal MRI group, comparing focal lesions (hippocampal sclerosis, n=16 or focal cortical dysplasia, n= 8 or other abnormalities, n=11) did not identify any particular lesional abnormality associated with higher levels of HMGB1 (data not shown). Furthermore, comparison within focal epilepsy between those with drug-resistant epilepsy (n=58 out of 65) and well-controlled epilepsy (n= 9 out of 20) did not identify a significant difference in HMGB1 levels (data not shown), likely due to small sample size.

ROC analysis showed that total HMGB1 level clearly separated drug-resistant from drug-responsive epilepsy (total ROC-AUC 0.99, P = 0.0001; sensitivity at 95% specificity: ROC-AUC 0.94 (95% CI 0.85–

0.98) with a cut-off HMGB1 concentration of 2.3 ng/ml) or healthy controls (AUC=0.99, p=0.0001; sensitivity at 95% specificity: ROC-AUC of 0.93 (95% CI 0.85–0.99) with a cut-off of HMGB1 concentration of 2.3 ng/ml) (**Fig. 1c**).

No significant association was identified between index seizure duration, seizure frequency (p=0.61) in the previous month or epilepsy sub-type (p=0.87) and baseline HMGB1 level.

Newly diagnosed epilepsy cohort. In patients at the time of diagnosis the baseline HMGB1 level in serum was 5.30 ± 4.60 ng/ml (mean±SD; n=28). No correlation was identified between blood HMGB1 level and the total pre-treatment seizure count (n=26, **Fig. 2a**), nor was there any correlation between HMGB1 and the number of days since last seizure at pre-treatment baseline (n=26, **Fig. 2b**). There was no significant difference between HMGB1 levels between those with normal and abnormal MRI scans (data not shown).

Discussion

We report that patients with long-standing, drug-resistant epilepsy had significantly higher HMGB1 levels in blood compared to patients with drug-responsive, well-controlled epilepsy or healthy controls. Previously, HMGB1 and TLR4 have been shown to be significantly elevated in peripheral blood following acute seizures in patients with epilepsy compared with healthy controls [8].

Nucleus-to-cytoplasmic translocation of HMGB1, a known mechanism for allowing cellular release, has been demonstrated in neurons and astrocytes in human tissue resected at surgery from drug-resistant epilepsy foci[2,9]. It is possible therefore that blood levels reflect brain-to-blood passage although one cannot exclude that circulating HMGB1 may also be derived from peripheral leukocytes. Additionally, HMGB1 is up-regulated in skeletal muscle inflammation and is released upon tissue injury [10], therefore suggesting a possible source being muscle due to generalised seizure activity; however, this would not explain the high levels of HMGB1 measured in patients with focal-only seizures. Notably, we found that blood HMGB1 levels were higher in long-standing, drug-resistant epilepsy patients with MRI abnormalities known to be associated with a greater risk of developing drug resistance [11], possibly reflecting more extensive reactive gliosis due to structural damage. There was no significant association with imaging abnormalities in either well-controlled or newly-diagnosed patients with epilepsy (however, only 5 out of 26 patients in this cohort had MRI scans reported as abnormal). Furthermore, comparison of focal epilepsy between those with drug-resistant epilepsy and well-controlled epilepsy did not identify a significant difference in HMGB1 levels, likely due to small sample size. Focal epilepsy predominates in the drug-resistant group, reflecting real-world practice. Indeed, generalized epilepsies, largely presumed to be genetic in origin, are generally easier to control on monotherapy. Focal epilepsies arise as a consequence of

varied and multifactorial lesional abnormalities and have a more complex response to therapy. Thus, the higher baseline levels of HMGB1 seen in drug-resistant epilepsy may also reflect the underlying brain pathology of focal epilepsy, but still serves as a potentially valuable biomarker regardless.

Different epilepsy types do not appear to significantly impact on HMGB1 levels, as reported in a recent study [12]. In particular, this study confirmed in independent patient cohorts that serum levels of HMGB1 are significantly elevated in drug-resistant patients vs controls. Notably, in the drug-resistant cohort there was no significant difference in serum HMGB1 levels between patients with symptomatic etiology (4.98 ± 2.90 ng/mL, n =15) and cryptogenic etiology (3.97 ±2.72 ng/mL, n =12, p =0.323). Additionally, no significant difference in serum HMGB1 levels was found among drug-resistant patients with focal onset seizures (4.58 ±2.88 ng/mL, n = 12), generalized onset seizures (3.46 ± 2.47 ng/mL, n = 7), and unknown onset seizures (5.40 ±3.03 ng/mL, n =8; p =0.526). In epileptic dogs, with regard to seizure etiologies, serum HMGB1 concentrations of idiopathic epilepsy and structural epilepsy were significantly higher than those of the healthy control dogs. However, there were no significant differences between the serum HMGB1 concentrations of idiopathic vs structural epilepsy dogs [13](P = .41).

The available data therefore support that higher levels of HMGB1 in serum of drug-resistant vs drug-responsive patients are unlikely to reflect the different epilepsy types or different seizure types but rather they likely reflect intrinsic propensity of the patient to respond or not respond to antiseizure medication.

Importantly, our data indicate that changes in blood HMGB1 do not solely reflect ongoing seizure activity, although repeated generalized seizure activity may give risk to low-level HMGB1 release and is consistent with low HMGB1 levels in those with well-controlled epilepsy. However, we report a lack of correlation between blood HMGB1 level and pre-treatment seizure count consistent with the lack of correlation between HMGB1 blood levels and the number of seizures in chronic epileptic rats with long-standing spontaneous seizures[14]. Moreover, should HMGB1 purely reflect seizure activity, then one would expect those patients with the most recent seizures to express higher levels of the biomarker. We did not find this to be case in our studies.

This proof-of-concept study shows that blood HMGB1 differentiates drug-resistant from well-controlled epilepsy, and that the blood levels are not merely determined by ongoing seizures, suggesting that HMGB1 reflects intrinsic pathogenesis of drug-resistant seizures. Thus, this novel evidence supports the hypothesis that blood HMGB1 might identify patients with the highest risk of developing drug-resistant epilepsy. Prospective clinical studies examining the prognostic value of blood HMGB1 at epilepsy diagnosis, along with elucidating the extent to which structural, focal abnormalities contribute to elevated HMGB1, are needed. Additionally, whether HMGB1 acts as a

biomarker for predicting response to therapy in patients treated with either available ASDs or new agents in development. This would be consistent with studies in a rat model of drug-resistant epilepsy which have shown that blood HMGB1 levels are good predictors of the therapeutic effects of both anti-inflammatory and anti-oxidant drugs[14,15]. Targeting inflammation may represent a novel therapeutic strategy for epilepsy, and circulating biomarkers able to demonstrate both target engagement and treatment response are of high value to guide drug discovery. Existing anti-inflammatory drugs could be repurposed towards drug-resistant epilepsy, at doses sufficient to achieve pharmacokinetic and pharmacodynamic parameters capable of modulating neuroinflammation. One notable example is the recent use of anakinra, the IL-1 receptor antagonist, to control unremitting seizures in patients with new onset refractory status epilepticus [16].

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Figure Legends

Figure 1. *HMGB1 levels in serum of drug-resistant and drug-sensitive epilepsy patients and healthy controls*

Panel (A): Dot blot (left) shows total HMGB1 levels in healthy controls (1.1 ± 0.07 ng/ml, n=74; light blue symbol) and patients with well-controlled epilepsy (1.2 ± 0.15 ng/ml, n=20; dark blue symbol) or drug-resistant epilepsy (8.7 ± 0.47 ng/ml, n=65; $p<0.01$; red symbol). Data are presented in each group as individual values as well as the mean value ± s.e.m.; **$p<0.001$ by one way ANOVA.

ROC analysis (right) of total HMGB1 discriminates (AUC 0.99) between patients with drug-resistant seizures and both healthy controls and those with well-controlled seizures.

Panel (B): Total HMGB1 levels in blood serum of drug-resistant epilepsy patients (mean ± SEM, n=65) with (n=35) or without (n=30) MRI abnormalities. *$p< 0.01$ by t-test.

Figure 2. *HMGB1 and pre-treatment seizures*

Lack of correlation between high mobility group box-1 (HMGB1) and the total pre-treatment seizure count reckoned at diagnosis (baseline visit, n=26; panel A) and the number of days since last seizure (panel B) in patients with newly diagnosed epilepsy.