Circulating damage marker profiles support a neuroprotective effect of erythropoietin in ischemic stroke patients

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Running head: EPO treatment reduces circulating damage markers

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ABSTRACT

The German Multicenter EPO Stroke Trial, investigating safety and efficacy of erythropoietin (EPO) treatment in ischemic stroke, had formally to be declared a negative study. Exploratory subgroup analysis, however, revealed that patients not receiving thrombolysis most likely benefited from EPO regarding clinical recovery - a result reproducing findings of the Göttingen EPO Stroke Study. The present work investigated whether the positive signal on clinical outcome in this patient subgroup is mirrored by respective post-stroke biomarker profiles. All patients of the German Multicenter EPO Stroke Trial non-qualifying for thrombolysis were included, if they (I) were treated per protocol and (II) had at least 2 out of 5 follow-up blood samples for circulating damage markers drawn (n=163). The glial markers S100B and GFAP and the neuronal marker UCH-L1 were measured by ELISA in serum of days 1, 2, 3, 4, and 7 post-stroke. All biomarkers increased post-stroke. Overall, EPO treated patients had significantly lower concentrations (area under the curve) over 7 days of observation as reflected by the composite score of all 3 markers (Cronbach's α=.811) and by UCH-L1. S100B and GFAP showed a similar tendency. To conclude, serum biomarker profiles, as outcome measure of brain damage, corroborate an advantageous effect of EPO in ischemic stroke. In particular, reduction in the neuronal damage marker UCH-L1 may reflect neuroprotection by EPO.

Introduction

Since 1998, erythropoietin (EPO) has proven its neuroprotective and neuroregenerative potential in nearly 200 preclinical studies, ranging from ischemia and neurotrauma to inflammation and neurodegeneration. Essentially all of the few clinical studies performed in the neuroscience field have yielded positive results with respect to EPO treatment effects (reviewed in (1, 2)). The first encouraging clinical study was the Göttingen EPO Stroke Study, showing beneficial outcome of ischemic stroke patients upon EPO (3).

Unfortunately, the respective follow-up study, the German Multicenter EPO Stroke Trial (*ClinicalTrials.gov Identifier: NCT00604630*), building on these positive results, turned out as formally negative trial, due to the unexpectedly large percentage of rtPA (recombinant tissue plasminogen activator) treatments with a high violation rate of thrombolysis contraindications (4). Patients with rtPA application despite prior anticoagulation had inferior outcome under additional EPO, whereas patients treated with rtPA *'lege artis'* did not have any disadvantage of EPO treatment (*www.epo-study.de*) (4). Potential mechanisms explaining a negative interplay between EPO and rtPA were recently reported in preclinical work (5, 6). In contrast, patients who did not receive rtPA likely benefited from EPO with a clinical course/outcome (NIHSS) comparable to that obtained in the first EPO stroke study (3, 4). In the absence of any other neuroprotective or neuroregenerative strategy available for stroke patients, this promising signal encourages further work along these lines.

Circulating biomarkers of brain damage are increasingly considered as additional outcome measures for stroke complementing clinical and imaging data. Among the markers selected for the present analysis, the glial damage markers S100B and GFAP (glial fibrillary acidic protein) have been in clinical use for many years, whereas the neuronal marker UCH-L1 (ubiquitin C-terminal hydrolase) has been integrated recently in the repertoire of stroke biomarkers. All these damage markers correlate well with clinical severity, course and outcome of brain injury (7, 8).

UCH-L1 is a highly abundant protein that resides in almost all neurons and averages between 1-5% of total soluble brain proteins. It has been suggested that UCH-L1 plays a critical role in the removal of excessively oxidized or misfolded proteins both during normal and neuropathological conditions (9-12). Based on this important neuronal function and its high specificity and abundance in the CNS, we have selected UCH-L1 here as a candidate biomarker for post-stroke brain injury and readout of neuroprotection.

S100B is a low-molecular weight glial protein of a multigenic family of calcium-binding proteins, highly specific to the nervous system and found in abundance in the astroglia compartment in the cerebral cortex, in peripheral Schwann cells, but also extra-neuronally in melanocytes, adipocytes and chondrocytes (13). In previous studies, we could show that S100B release was associated with stroke severity and clinical outcome (14). S100B has also been postulated to be a marker of generalized blood-brain barrier dysfunction, rather than of specific glia damage only (15).

Glial fibrillary acid protein (GFAP) is a monomeric filament protein localized to astrocytes in the brain. GFAP is involved in various neuronal processes, including maintenance of the blood-brain-barrier (for review see (16)). Increased serum concentrations of GFAP were described following ischemic stroke and traumatic brain injury, and to correlate with clinical severity and outcome (17, 18).

About 10 years ago, we argued that molecular markers of brain damage might be a useful tool in translational stroke research, and that the analysis of the release patterns of biomarkers might be a promising strategy to evaluate neuroprotective approaches in stroke treatment (19). Here we report post-stroke biomarker profiles of an exploratory subgroup comprising per-protocol treated ischemic stroke patients of the German Multicenter EPO Stroke Trial who did not receive rtPA.

Patients and Methods

Patients

The present predefined exploratory subgroup analysis is based on all patients of the randomized, double-blind, placebo-controlled German Multicenter EPO Stroke Trial (4) who fulfilled the following requirements: They (I) were treated per-protocol, (II) had not received rtPA, and (III) had at least 2 out of 5 follow-up blood samples for circulating damage markers drawn, resulting in a total of n=163 patients (exclusion of n=3 due to missing serum samples). Main inclusion criteria were acute ischemic stroke in the middle cerebral artery territory leading to a score ≥4 in the National Institutes of Health Stroke Scale (NIHSS).

Study Intervention

Intravenous infusion of recombinant human EPO (Epoetin-alpha, 40,000IU) or placebo was given within 6h after symptom onset (day 1) and repeated 24h and 48h later (4). The dose was chosen according to the previous EPO study (3).

Biomarker Assays

Blood for biomarker analysis was drawn on days 1, 2, 3, 4, and 7. Serum was stored at -80°C. ELISAs of S100B, GFAP and UCH-L1 were performed blindly, i.e. without clinical information, using antibodies from Banyan (Alachua, FL, USA), Sigma (St.Louis, USA) and Dako (Carpinteria, CA, USA).

Statistical Analysis

For each marker, a linear regression based multiple imputation (10 iterations) model of missing data (UCH-L1 5.8 %; S100B 6.5 %; GFAP 20.6% missing) was applied, if at least 2 out of 5 values per subject were present, resulting in n=163 subjects for UCH-L1 and S100B, and n=154 for GFAP. All per-protocol treated non-rtPA individuals not meeting this criterion were excluded from further analysis (UCH-L1 and S100B n=3; GFAP n=12). Areas under the curve (AUCs) for every marker were determined for each imputation matrix by the composite trapezoidal rule for numerical integration. The pooled AUC represents the mean of the 10 AUC matrices per marker. Two composite scores were calculated reflecting the mean of the z-standardized pooled AUC values for UCH-L1, S100B and GFAP (Cronbach's α=.811) and for S100B and GFAP (Cronbach's α=.755). For a total of n=9 individuals, the composite scores had to be based on the z-standardized pooled AUC values for UCH-L1 and S100B only. Mann-Whitney U-

Tests (2-tailed) and Chi-square tests or Fisher's exact test were used for intergroup comparisons. Analysis of variance for repeated measures was applied to compare EPO versus placebo with respect to delta NIHSS (NIHSS at baseline - NIHSS day 90). Analysis of covariance with NIHSS score at baseline as covariate compared both groups with respect to pooled single marker AUC values and AUC composite scores. Further, a correlation analysis (Pearson) of delta NIHSS and UCH-L1 AUC was performed. Data are presented as mean±SD in text/tables and median or mean±SEM in figures.

Results

Patient characteristics at inclusion were well balanced between EPO and placebo groups in all important baseline variables, representative of a typical stroke population (Table 1). Biomarker profiles in serum displayed the expected increases between days 2 and 4 post-stroke, with peak time points varying considerably among different markers and individual patients (Figure 1).

The clinical course of included per-protocol treated non-rtPA patients (n=163) demonstrates a slightly better outcome of the EPO compared to the placebo group (mean delta NIHSS of 5.3±5.3 in EPO versus 3.3±6.5 in placebo; p=.039) (Figure 2A). As best estimate of total increase in circulating damage marker concentrations, AUCs were calculated for each marker in all patients. AUCs, corrected for NIHSS day 1 (severity of stroke symptoms upon inclusion, i.e. before any study drug treatment), turned out to be significantly lower in EPO versus placebo patients for UCH-L1 and showed a similar tendency for S100B and GFAP (Figure 2B).

To make use of the complete biomarker information, a z-standardization of the AUC scores for each marker was performed. AUC composite scores of the 2 glial markers and of all 3 biomarkers were calculated. The internal consistency of these composite scores turned out to be sufficiently high (n=154; Cronbach's alpha: .755; and n=154; Cronbach's alpha: .811, respectively) to justify their use as composites. Figure 2C illustrates z-standardized biomarker AUC levels for all single markers and the 2 composites showing that all 3 biomarkers discriminate between EPO and placebo groups, with UCH-L1 as single marker and the 3-marker composite score reaching statistical significance. Correlation coefficients of delta NIHSS and UCH-L1 AUC were found to be significant for both treatment groups with a numerically higher value in EPO patients (Figure 2D). This again emphasizes the neuroprotective property of EPO in stroke.

Discussion

The present exploratory subgroup study builds on the observation that stroke patients of the German Multicenter EPO Stroke Trial, non-qualifying for rtPA treatment, seemed to have a better clinical course/outcome under EPO compared to placebo (4). This observation is further supported here by respective stroke biomarker profiles. Especially the blunted increase in the neuronal damage marker UCH-L1 under EPO points to neuroprotection. The results obtained with this marker may actually suggest its extended use as a surrogate marker of stroke severity in future neuroprotection trials.

Similar to the first EPO stroke study (3), the S100B increase tended to be lower in EPO patients but failed to reach statistical significance here. The third evaluated marker, GFAP, turned out to be the least responsive one in the present analysis, perhaps due to the necessity (insufficient sample volume left) to impute circa 20% of missing data (as compared to only around 6% for S100B and UCH-L1). The composite score of both glial markers, S100B and GFAP, produced a 'near-significant' result. The composite of all 3 markers, though different between treatment groups, does at first view not add to the information obtainable with the neuronal marker UCH-L1 alone. However, both composite scores reveal that all contributing markers, be it of glial or neuronal origin, essentially behave synergistically.

Conclusions

Stroke is a very common, devastating and frequently severely disabling condition with only thrombolysis and supportive measures presently available for treatment. The former still reaches just a small percentage of patients, and the increasing violation of rtPA contraindications (as experienced also in the German Multicenter EPO Stroke Trial) reflects desperation and fatalism of treating personnel in the absence of alternative therapeutic options. Importantly, stroke patients are extremely heterogeneous with respect to genetic and environmental predisposing factors including comorbidities, explaining why huge effects of novel treatment strategies can never be expected over all patients. Therefore, even the slightest signal of benefit of neuroprotective treatment strategies has to be vigorously pursued. In this regard, the course of circulating brain damage markers upon EPO - in association with the documented clinical improvement - should encourage further work on EPO or EPO variants/analogues in ischemic stroke patients that are not eligible for thrombolysis.

Acknowledgements

This study was supported by the Max Planck Society and Banyan Biomarkers, Incorporated.

Disclosures

HE holds/has submitted patents on EPO for stroke, schizophrenia and MS. Jackson Streeter, Kevin Wang, Ron Hayes, and Andreas Jeromin are employees of Banyan Biomarkers, Incorporated, a company developing biomarkers for brain diseases. There are no other disclosures/conflicts of interest to be declared by any of the authors that would be of any relevance to the topic of the manuscript.

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Legend to Figure 1

Course of circulating brain damage markers after ischemic stroke

All 3 biomarkers measured in serum over time increase after stroke (placebo white - left panels; EPO black - right panels). Note the logarithmic scale of presentations. Medians given.

Legend to Figure 2

Biomarkers substantiate the positive signal on clinical outcome of EPO compared to placebo patients

(A) EPO patients show improved clinical outcome (NIHSS) after stroke as compared to the placebo group. **(B)** AUC mean±SEM values and **(C)** AUC z-standardized values demonstrate differences in biomarkers post-stroke between EPO and placebo patients. **(D)** Delta NIHSS and UCH-L1 AUC correlate in both treatment groups with a numerically higher correlation coefficient in EPO patients.

Table 1. Patient Characteristics on Inclusion

	Patients included in subgroup-analysis (N=163)		
Variable	**EPO (N=76)**	**Placebo (N=87)**	**p-value**
Age, years, mean±SD	71.14±11.45	71.37±10.78	**1.00***
Sex, male/female ratio (%)	40/36 (52.6/47.4)	44/43 (50.6/49.4)	**.875**
Number of deaths (%)	6 (7.9)	8 (9.2)	**1.00**
Hemisphere, N (%)			
Left	35 (46.1)	40 (46.0)	
Right	41 (53.9)	46 (52.9)	**.879**
Both	0 (0.0)	1 (1.1)	
Stroke subtype, N (%)			
Cardiogenic embolism	34 (44.7)	42 (48.3)	
Arterial embolism	18 (23.7)	17 (19.5)	
Large artery occlusion	13 (17.1)	14 (16.1)	**.985**
Paradox embolism	2 (2.6)	2 (2.3)	
Lacunar infarction	5 (6.6)	6 (6.9)	
Unknown	4 (5.3)	6 (6.9)	
Prior anticoagulation, N (%)			
No	42 (55.3)	45 (51.7)	
Yes	34 (44.7)	41 (47.1)	**.601**
Unknown	0 (0.0)	1 (1.1)	
Hypertension, N (%)			
No	18 (23.7)	22 (25.3)	
Yes	58 (76.3)	62 (71.3)	**.246**
Subclinical/borderline	0 (0.0)	3 (3.4)	
Unknown	0 (0.0)	0 (0.0)	
Diabetes, N (%)			
No	58 (76.3)	60 (69.0)	
Yes	15 (19.8)	24 (27.6)	**.503**
Subclinical/borderline	3 (3.9)	3 (3.4)	
Unknown	0 (0.0)	0 (0.0)	
NIHSS			
Mean±SD (range)	12.08±5.90 (4-27)	11.47±5.52 (4-27)	**.539***
MRI diffusion-weighted imaging, cm^3			
Mean±SD (range)	34.84±44.11 (0-186)	42.39±65.52 (0.2-298)	**.688***
MRI FLAIR, cm^3			
Mean±SD (range)	4.06±12.10 (0-77)	2.26±4.9 (0-23)	**.895***
Time to treatment, minutes			
Mean±SD (range)	275.32±79.10 (42-442)	278.98±65.85 (78-485)	**.977***

* P-values from group comparison by Mann-Whitney U-Test. All other p-values obtained from two-sided Chi-square test or Fisher's exact test