Release of Glial Tissue–Specific Proteins After Acute Stroke: A Comparative Analysis of Serum Concentrations of Protein S-100B and Glial Fibrillary Acidic Protein

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Background and Purpose—This study was aimed at the comparative analysis of serum concentrations of glial fibrillary acidic protein (GFAP) and protein S-100B in patients with acute stroke.

Methods—We investigated 32 patients with stroke symptoms consistent with cerebral ischemia in the anterior territory of vascular supply. Venous serial blood samples were taken after admission to the hospital and during the first 4 days after onset of stroke. Evaluation of lesion topography and volume of infarcted brain area was based on cranial CT data. The patients’ clinical status was consecutively evaluated by the National Institutes of Health Stroke Scale (NIHSS) and the Barthel Index score at discharge from the hospital.

Results—Protein S-100B and GFAP release was found to be significantly correlated \( r = 0.96; P < 0.001 \). The release of both biochemical markers was associated with the volume of brain lesions \( r = 0.957, P < 0.0001; \) GFAP: \( r = 0.955, P < 0.0001 \) and the neurological status at discharge from the hospital \( r = 0.821, P = 0.0002 \); GFAP: \( r = 0.717, P = 0.0003 \). The highest correlation between both S-100B and GFAP serum concentration and Barthel score was calculated at the last time of blood sampling, 4 days after stroke onset \( r = 0.821, P < 0.001; \) GFAP: \( r = 0.655, P < 0.001 \). The release of both astroglia derived proteins differed between different subtypes of stroke. GFAP was found to be a more sensitive marker of brain damage in patients with smaller lacunar lesions or minor strokes.

Conclusions—Our results indicate that postischemic release patterns of GFAP and S-100B protein may allow insight into the underlying pathophysiology of acute cerebral infarcts and may be used as a valuable tool of clinical stroke treatment.

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Key Words: glial fibrillary acidic protein ■ nerve tissue protein S-100 ■ outcome ■ stroke

During the past decade the analysis of neurobiochemical markers of brain damage has attracted increasing attention in a variety of central nervous system disorders. Because of commercial availability and detectability in peripheral blood, neuron-specific enolase (NSE) and protein S-100B were the biochemical markers of brain damage studied most often in clinical settings. Both proteins are considered specific markers of brain damage after stroke, traumatic brain injury, cardiac surgery under cardiopulmonary bypass condition, or cardiac arrest.

Meanwhile, there exist a considerable number of studies investigating the release patterns of protein S-100B into cerebrospinal fluid (CSF) or peripheral blood after acute stroke and their association with the volume of lesion, clinical status, and functional outcome. Poststroke S-100B serum concentrations were reported to correlate significantly with the size of infarcted brain areas, and the release pattern of the brain-originated protein was interpreted to mirror the underlying pathophysiology of acute stroke or traumatic brain injury. Furthermore, several clinical studies could establish a significant association between early serum concentrations of S-100B and the clinical and/or functional outcome after stroke.

Protein S-100B forms part of a large and diverse family of binding proteins predominantly found in astrocytes and Schwann cells. The biological properties of the protein are not fully understood to date, but there is evidence that S-100B modulates complex neuronal-glial interactions. A variety of experimental findings suggest both a detrimental (induction of neuronal cell death) and a beneficial (induction of reactive synaptogenesis and plasticity processes) potential of protein S-100B dependent on concentration and time elapsed since brain injury. Protein S-100B, however, is expressed not only in brain tissue but also in a variety of other...
cell types, under both physiological and pathological conditions. Expression of protein S-100B has been observed in white and brown fat, skin and skeletal muscle tissue, melanoma or glioblastoma cells, as well as in patients treated for a longer time with β-adrenergic agonists or phosphodiesterase inhibitors. Although S-100B expression in the latter conditions was far below the activity measured after acute central nervous system disorders, the brain specificity of S-100B release was questioned by a number of investigators. Consequently, there was a demand for neurobiochemical markers highly specific for astroglial brain tissue, and glial fibrillary acidic protein (GFAP) was considered a high-priority candidate. GFAP is a monomeric intermediate filament protein expressed almost exclusively in astrocytes, where it represents the major part of the cytoskeleton. Several studies have shown that CSF concentrations of GFAP might be elevated in normal pressure hydrocephalus, dementia, but apart from preliminary clinical data in a technical brief, there is no systematic study on GFAP serum concentrations in stroke or other brain disorders.

The present study aimed at a comparative analysis of GFAP and S-100B serum concentrations and release patterns in patients after acute stroke. Our main objectives were (1) to analyze the poststroke correlation of both neurobiochemical markers of glial brain tissue, (2) to evaluate the association with stroke subtype and volume of infarcted brain areas, and (3) to contrast the predictive value of both proteins with respect to the early neurological and functional outcome after acute stroke. To reach these goals we reanalyzed a subsample of a well-characterized group of patients presented in a previous study, with acute stroke symptoms corresponding to ischemia in the anterior territory of vascular supply.

Subjects and Methods

Subjects

From a consecutive series of patients admitted to the Neurology Department at Magdeburg University, we included 32 patients with infarcts in the anterior circulation system. Exclusion criteria were hemorrhagic stroke or ischemic infarction in the posterior territory of vascular supply and concomitant brain disorders potentially interfering with S-100B or GFAP release. All patients gave informed and written consent to participate at the study. According to the Bamford classification scheme, 5 patients (15.6%) presented with lacunar infarcts, 15 patients (46.9%) with partial anterior circulation infarcts, and 7 patients (21.9%) with total anterior circulation infarcts; in 5 patients (15.6%) stroke subtype could not be classified. Stroke etiology, according to the TOAST criteria, was cardioembolism in 9 patients (28.1%), small-artery occlusion in 7 patients (21.9%), and large-artery atherosclerosis in 7 patients (21.9%). Origin of stroke was not identifiable in 9 patients (28.1%). The mean ± SD time interval between the onset of stroke symptoms and admission to the stroke unit was 8.4 ± 5.9 hours. Demographic, clinical, and neuroradiological data are shown in the Table.

Methods

Neurobiochemical Analysis

Venous blood samples were taken at admission (mean ± SD time after stroke onset, 8.4 ± 5.9 hours), and at the first (20.4 ± 6.8 hours), second (44.0 ± 6.7 hours), third (68.1 ± 7.6 hours), and fourth days (92.7 ± 8.0 hours). Blood was allowed to clot, and after centrifugation within 30 minutes (1000g, 10 minutes), serum was stored at −78°C for later analysis. Protein S-100B was analyzed using a commercially available monoclonal 2-site immunoluminometric assay (Sangtec 100). The assay kit measures the β-subunit of the protein as defined by 3 monoclonal antibodies (SMST 12, SMSK 25, and SMSK 28). Antibody-coated polystyrene tubes serve as solid phase in which the coated antibody reacts with the S-100B protein in the sample. Unbound material is removed by a washing step. During a second incubation the tracer antibody binds to the immobilized S-100B, and the nonreactive tracer is removed by a second washing. The anti-S-100B tracer conjugate contains a covalently bound isoluminol derivative. For detection, the isoluminol is oxidized by alkaline peroxide followed by light emission. The whole procedure was performed in an automated LIA-mat System 300, with a detection threshold below 0.02 µg/L. The range of S-100B serum concentrations in 95% of healthy subjects is reported to be <0.12 µg/L. GFAP measurement is based on an in-house enzyme-linked immunoassay. For each assay a microtiter plate is coated with rabbit GFAP antibody. After a 5-fold washing cycle with phosphate buffer pH 7.4, containing 0.05% Tween 20, a blocking agent is applied. After washing, either human GFAP antigen or serum (in duplicate)
are added and incubated for 2 hours. After washing, diluted mouse anti-human GFAP is added and incubated for another hour. After a further washing cycle, a diluted biotinylated rabbit anti-mouse IgG is applied and incubated for 1 hour. After washing, a diluted peroxidase-conjugated streptavidin solution is added and incubated for 30 minutes. After a final washing cycle, 100 μL of a freshly prepared tetramethylbenzidine solution is added and incubated for 15 minutes in the dark. The reaction was stopped by adding 100 μL 2N H₂SO₄, and absorbance was read at 450 nm on an ELISA reader (Titertek ICN). The upper limit of GFAP in serum of healthy subjects was measured at <0.3 μg/L.

Analysis of GFAP was performed at the University Hospital at Nijmegen, the Netherlands, and S-100B was measured at Magdeburg University, Magdeburg, Germany. The investigators were blinded to clinical and neuroradiological data.

Neuroradiological Assessment
All neuroradiological examinations were based on cranial CT imaging. Scans were performed briefly after admission (mean±SD 6.0±6.3 hours after infarction) in standardized slices without contrast enhancement and were repeated within the first week (mean 64.8±51.1 hour after infarction, n=27). Imaging data of all subjects were analyzed using the public domain NIH Image program (developed at the US National Institutes of Health and available via Internet at http://rsb.info.nih.gov/nih-image). All CTs were evaluated with respect to lesion topography and territories of vascular supply, and volume of lesions was calculated in all scans showing a clearly demarcated infarct area (n=28). Neuroradiological data analysis was performed independently by 2 members of our group, 1 of whom was blinded to all other data. Data on reliability and interrater agreements are given elsewhere.

Neurological and Functional Assessment
All patients underwent a standardized neurological examination on admission, at the first and fourth days after onset of stroke symptoms, and at discharge from the hospital. The neurological status was quantified (by M.T.W.) with use of the National Institutes of Health Stroke Scale (NIHSS²⁹), and the functional outcome at discharge was rated with a modified Barthel Index score by experienced neurologists.

Statistical analyses were performed with the SPSS 8.0 program package (SPSS, Inc) and the MedCalc 5.0 statistical software (MedCalc Software).

Results
Neurobiochemical and Neuroradiological Data
We found no significant association between the release of either biochemical marker and sex or age of patients. Figure 1 shows the release patterns of protein S-100B and GFAP with respect to all patients. Mean GFAP serum concentrations at the time of admission (8.4 hours after stroke onset) already exceeded the reference value of healthy control subjects, whereas protein S-100B started to reach critical values 20 hours after onset of stroke symptoms. Both protein concentrations continuously increased until the fourth day after stroke onset. This increase with respect to the whole patient group, however, has to be attributed to subjects with large total anterior circulation infarcts (n=7; mean volume of lesions, 112.4±10³ mm³). Figure 2 presents the release patterns of both markers separately for small lacunar, partial, and total anterior circulation infarcts. In lacunar infarcts the GFAP serum concentrations were elevated at the time of admission and thereafter continuously decreased. S-100B serum concentrations did not reach values above the cutoff level. Mean serum concentrations in partial anterior circulation infarcts constantly remained above the cutoff value (GFAP) or continuously increased with pathological values starting 2 days after stroke (S-100B). In total anterior circulation infarcts, the serum concentrations of both proteins were found to be elevated at admission and continuously increased. Four days after the acute stroke event, peak concentration of GFAP was measured at 28-fold above the cutoff value, and maximal concentration of protein S-100B reached 18-fold elevated values.

The area under curve (AUC) values of GFAP and S-100B were significantly correlated (Pearson’s r=0.96, P<0.0001). Serum concentrations at the different sampling points, however, were found to be significantly correlated not before the second day after stroke (r=0.837, P<0.001; day 3: r=0.961, P<0.0001; day 4: r=0.961, P<0.0001).

In all but 4 patients, demarcated areas of brain infarction in the anterior circulation territory that corresponded to the acute stroke symptoms could be identified. The infarcts mostly involved temporal and parietal brain areas. Accordingly, they were mainly supplied by medial and posterior branches of the middle cerebral artery and the lenticulostriate arteries. Two patients exhibited anterolateral thalamic lesions in the tuberohalamic artery territory of vascular supply. Mean absolute lesion volume was calculated 34.2±10³ mm³ (±72.2). Volume of lesions and serum concentrations of both proteins were significantly correlated (S-100B, r=0.957, P<0.0001; GFAP, r=0.955, P<0.0001). Protein S-100B values were found to significantly correlate with the volume of brain infarction, starting at the time of admission to the hospital (r=0.676, P=0.0003; day 1: r=0.940, P=0.0002; day 2: r=0.949, P=0.0002; day 3: r=0.969, P=0.0002; and day 4: r=0.892, P=0.0007). Significant correlations between
GFAP serum concentration and volume of lesion could be observed beginning with the second day after stroke ($r=0.799$, $P=0.0004$; day 3: $r=0.968$, $P=0.0003$; and day 4: $r=0.957$, $P=0.0004$).

Release of Neurobiochemical Markers and Neurological and Functional Status
With respect to all patients, NIHSS scores showed a continuous and significant improvement between admission and discharge (Friedman test: $\chi^2=30.2$, df=3, $P=0.0002$). Serum concentrations of S-100B and GFAP correlated significantly with NIHSS-scores at any time of blood sampling. The numerically highest and most significant association was found between S-100B and GFAP AUC values and the neurological status at discharge from the hospital (S-100B: $r=0.821$, $P=0.0002$; GFAP: $r=0.717$, $P=0.0003$). To obtain information on the predictive value of both proteins, we dichotomized the NIHSS scores at the fourth day after stroke onset (the last day of blood sampling) according to the criteria proposed by the TOAST investigators. An NIHSS score of $\leq 6$ was taken as an indicator of good recovery, whereas a score $>6$ was associated with a higher probability of poor outcome. Figure 3 shows a comparison of receiver operating characteristic (ROC) curves of S-100B and GFAP AUC values with respect to the forecast of a good (n=19) or poor (n=13) outcome. Areas under the ROC curves and the respective 95% confidence intervals indicate that both proteins do have a significant predictive value according to the separation of patients with a good or poor prognosis. The protein S-100B AUC value was calculated slightly higher than the GFAP AUC value.

Figure 2. Release patterns of protein S-100B (right) and GFAP (left) in lacunar infarcts, partial anterior circulation infarcts, and total anterior circulation infarcts (means±1 SEM); the scaling of S-100B and GFAP concentrations was adjusted according to the upper limit of the respective reference range of 95% of healthy controls (shaded areas).

Figure 3. Comparison of ROC curves of GFAP (solid line) and S-100B (dotted line) AUC values. Outcome prediction was based on NIHSS scores at the fourth day after stroke (NIHSS $\leq 6$ indicates a good outcome [n=19] and NIHSS $>6$ a poor outcome [n=13]). The graph shows the true-positive rate in function of the false-positive rate at different cutoff points.
than the respective GFAP AUC value (0.700; 95% CI 0.51 to 0.85), but the areas under the ROC curves did not differ significantly (P = 0.351).

Functional outcome (Barthel score) at discharge from the hospital was significantly associated with the release of both proteins (S-100B AUC: r = 0.612, P < 0.001; GFAP AUC: r = 0.564, P = 0.001). The highest correlation between both S-100B and GFAP serum concentrations and Barthel scores was calculated at the last time of blood sampling, 4 days after stroke onset (S-100B: r = 0.621, P < 0.001; GFAP: r = 0.655, P < 0.001).

Release Patterns of S-100B and GFAP and the Clinical Course of Stroke

Figure 2 demonstrates that the release patterns of S-100B and GFAP depend on the size of infarcted brain area and significantly differ in lacunar infarcts, restricted cortical infarcts, and large anterior circulation infarcts. Figure 4 shows the release patterns of both proteins in 3 patients representing different clinical courses after stroke. Stroke in progression (case 1) was associated with a continuous increase of both neurobiochemical markers peaking at the third or fourth day after onset of stroke symptoms. Generally, GFAP peak levels exceeded the cutoff value to a higher extent compared with the maximal S-100B concentration. In completed stroke associated with a good recovery (case 2), peak values were found at the second day after stroke, followed by a continuous decrease. GFAP peak level exceeded the cutoff value up to 15-fold, whereas S-100B serum concentrations were found to be only slightly elevated. Patients with prolonged but fully reversible neurological deficits without demarcated brain lesion (case 3) showed no significant S-100B release. The initial GFAP serum concentrations, however, were found to be significantly elevated. This increase was followed by a rapid decline during the next 3 days.

Discussion

This study presents the first systematic approach to a comparative investigation of GFAP and S-100B serum concentrations after acute stroke. The release of both markers was found to be significantly correlated, and S-100B as well as GFAP poststroke serum concentrations were associated with
S-100B values and their highly significant correlation with GFAP release give evidence that S-100B serum concentrations indeed reflect damage to astrocytes. Interestingly, an analysis of the association between serum concentrations of both proteins at the different sampling times showed that a significant intercorrelation could not be observed before the second day after stroke. The lack of a significant correlation between GFAP and S-100B at the early stage after stroke has to be attributed to the finding of relatively higher initial GFAP serum concentrations. At admission to the hospital, 39% of all patients presented GFAP serum concentrations above the cutoff value, but only 19% of all patients showed S-100B serum concentrations exceeding the respective reference range.

We found significant correlations between postischemic GFAP and S-100B release and both neurological and functional status at discharge from the hospital. The literature on the association between S-100B release and the clinical status of stroke patients is heterogeneous, a fact that can be attributed mainly to different techniques of protein analysis and a broad range of instruments applied for the assessment of the neurological status and functional outcome after stroke. Büttner and coworkers reported a significant association of S-100B concentrations and neurological status only at the time of admission to the hospital, whereas other investigators also found significant correlations between S-100B values and the clinical and/or functional outcome.

As far as we know, the study of Aurell and coworkers was the first and only systematic approach to the analysis of both astroglial proteins in stroke patients. This study, however, had no clinical influence because the technique for protein analysis allowed the detection of GFAP and S-100B only in CSF. The data obtained from CSF samples, however, were highly consistent with the results of the present study. Aurell et al assigned the higher clinical significance to the analysis of GFAP. This statement was based mainly on the higher sensitivity of GFAP in small infarcts compared with the respective predictive value of protein S-100B. The results of the present study on poststroke serum concentrations of both proteins generally confirm the data from Aurell and colleagues. The higher sensitivity of early GFAP serum concentrations is corroborated by the GFAP and S-100B release pattern in patients with minor stroke. In patients with acute signs of ischemic stroke completely reversible within a few days, the initial GFAP values were measured as highly elevated, whereas S-100B concentrations were found to be far below the threshold or not measurable in serum samples.

The difference in temporal patterns of S-100B and GFAP release may be attributed to a different molecular biology of both proteins, which also might be mirrored in different release patterns in pathological conditions such as focal ischemia. Protein S-100B forms part of a large family of EF-hand Ca\(^{2+}\)-binding proteins, the cellular synthesis of which has been localized predominantly in astroglial and Schwann cells. The protein is involved in the differentiation of cytoskeletal structures and Ca\(^{2+}\)-dependent cellular information processing. Both necrotic cell death of astrocytes (leading to a leakage of protein S-100B from cytosol to the extracellular space) and breakdown of membrane integ-
rity in the penumbra zone of focal infarcts (due to cytotoxic and vasogenic edema) may result in a significant increase of S-100B serum concentration. Animal experiments and studies on cell cultures, however, indicate that protein S-100B not only reflects glial cell function but also modulates complex neuronal-glial interactions. Hu and coworkers demonstrated that S-100B treatment of astrocyte-neuron cocultures induces neuronal cell death via a nitric oxide–dependent pathway, a result that could not be replicated in the absence of astrocytes. Furthermore, astrocyte S-100B expression is also upregulated during lesion-induced sprouting and reactive synaptogenesis in adult rats. This finding suggests a major role of S-100B in brain repair mechanisms and plasticity. Glial fibrillary acidic protein is a 50-kDa intermediate filament protein that is expressed almost exclusively in astrocytes. The protein represents the major part of the astrocyte cytoskeleton, and it is required for the stable formation of astrocytic processes in response to neurons. Animal data indicate that transient or permanent suppression of cerebral blood supply, as well as photochemically induced focal cortical ischemia, result in a widely distributed upregulation of GFAP expression. A significant increase of GFAP immunoreactivity was observed 2 hours after reduced plasma perfusion following embolic MCA occlusion. The molecular biology of GFAP, however, remains largely unknown. Pekny and coworkers recently demonstrated that the genetic removal of GFAP is associated with an increase of the intracellular glutamine concentration. Thus, postischemic upregulation of GFAP expression may correlate with a decreased intracellular glutamine synthase activity. These data suggest a major role of GFAP in the control of the poststroke glutamine metabolism. Recent animal data also suggest an interaction between GFAP and protein S-100B. Seven days after postischemic reperfusion, Martinez and coworkers found only few GFAP positive cells in the rat hippocampus and cerebellum. This poor GFAP activity was interpreted as a consequence of the inhibition of GFAP polymerization by protein S-100B. GFAP depolymerization, however, seems to be part of a long-term adaptive response of astrocytes to ischemically induced metabolic alterations and neuronal death.

We present here for the first time a comparative analysis of the postischemic release patterns of GFAP and protein S-100B in serum. Taken together, both astroglia-derived proteins showed a significant association with the morphological and clinical consequences of acute stroke. The comparative analysis of the poststroke kinetics of GFAP and S-100B may allow valuable insight into the underlying pathophysiology of acute cerebral infarcts and may possibly give a hint of potential brain repair mechanisms. The present data also suggest that the postischemic GFAP and S-100B release may be a useful tool of monitoring and evaluating therapeutic interventions such as neuroprotective drug treatment, which is expected to be a major part of future stroke treatment.

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