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Questioning the role of actinfree Gc-Globulin as actin scavenger in neurodegenerative central nervous system disease: Relationship to S-100B levels and blood–brain barrier function

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ABSTRACT

Introduction: Preliminary studies report on significantly higher levels of the major cytoskeleton protein actin in CSF of patients with neurodegenerative conditions and that the dynamics of these levels obviously correlates with disease progression and clinical disability. One of the primary functions of actinfree Gc-Globulin is to bind and neutralize extracellular monomeric actin, released into the circulation by necrotic or ruptured cells, and thus ameliorating the clinical outcome in situations of severe organ damage.
Aim and methods: This is the first study to investigate actinfree Gc-Globulin and S100-B levels (as reliable marker of neurodegeneration) in paired CSF and serum samples of patients with multietiological CNS diseases.
Results: 42% of all patients with CNS disease displayed serum concentrations of actinfree Gc-Globulin above the established reference range. CSF concentrations of actinfree Gc-Globulin and S100-B were positively correlated with the severity of blood–brain barrier (BBB) dysfunction. Furthermore, patients with severe BBB dysfunction presented a higher percentage of intrathecal synthesis of actinfree Gc-Globulin compared to patients with mild to moderate dysfunction and to patients with normal BBB function. Representative longitudinal data from selected patients demonstrated an inverse behaviour of actinfree Gc-Globulin and S100-B CSF concentrations, suggesting a consumption of the actin scavenger capacity of Gc-Globulin in times of increased neuronal damage. This presumption was supported by the fact that those conditions associated with a severe neuronal damage, in particular CNS trauma, and highest S100-B concentrations simultaneously displayed lowest actinfree Gc-Globulin levels, and thus residual actin binding capacity of Gc-Globulin.
Conclusion: In summary, our data propose a function of actinfree Gc-Globulin also in the clearance of actin filaments from CSF of patients with neuronal damage. However, active recruitment of hepatic derived actinfree Gc-Globulin to the site of CNS injury is not observed. Much more, BBB leakage enables extraneuronally synthesized actinfree Gc-Globulin to extent its scavenger capacity for actin also to the subarachnoidal space. Furthermore, intrathecal synthesis of actinfree Gc-Globulin seems to be increased in patients with severe neurodegeneration.

1. Introduction

The axonal cytoskeleton of the neuron is a highly regulated system that plays a central role in maintaining the integrity of axons. The physiological functions of axonal cytoskeleton are dependent upon several interconnected filaments that primarily consist of actin microfilaments (6 nm in diameter), L-neurofilaments (10 nm) and microtubules (23 nm) [1]. Collectively, these proteins control axonal shape and caliber, maintain axonal transport of nutrients and organelles, define specialized membrane domains and regulate growth and focal adhesions [2–4]. Actin, one of the main proteins of the cytoskeleton, also plays an important role in axonal growth and guidance, in the formation and elongation of neuritis and in a variety of other biological responses [5].

Preliminary reports suggest that some cytoskeletal proteins may be detected in the cerebrospinal fluid (CSF) from patients with neurodegenerative conditions and that these proteins may serve as useful confirmatory markers of neurodegeneration or progression of CNS disorders [6]. For example, high levels of actin or its regulatory proteins have been detected in the CSF form patients with Alzheimer's disease [7,8]. Furthermore, significantly higher levels of the three major cytoskeleton proteins described above have recently been found in the CSF of MS patients compared to healthy and neurological controls and were obviously associated with progressive disease and clinical disability [9].

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Gc-Globulin is known as a multifunctional plasma protein of a molecular mass of 51–58 kD, which belongs to the albumin gene family predominantly synthesized in the liver. Its primary function is to serve as a carrier protein for vitamin D and its plasma metabolites. Furthermore, it is able to scavenge and neutralize extracellular monomeric actin (G-actin) released by necrotic or ruptured cells [10–15]. Thus, low total and actinfree Gc-Globulin concentrations, the latter being an index of residual actin-scavenging capacity, have been demonstrated to be prognostic markers in situations of severe organ damage, such as fulminant hepatic failure [16–19], acetaminophen (paracetamol) overdose [20], multiple trauma [21], and multiple organ failure [22–24].

This is the first study to investigate actinfree Gc-Globulin levels in CSF as well as in paired serum samples of patients with multi-etiological CNS diseases. The investigation is based on the hypothesis that the actin scavenging function of actinfree Gc-Globulin may be of considerable importance in ameliorating the overall clinical outcome of CNS pathologies, possibly contributing to reduce the onset of secondary brain damage.

2. Materials and methods

2.1. Study group

A total of 155 patients, 91 male, 64 female, age 52±21 years (mean±SD, range 1–86) with multietiological CNS diseases, presenting at the Neurological Emergency Unit, RWTH University Hospital Aachen, Germany, were included in this study.

At admission, advanced surgical residents under consultant supervision carried out a brief neurological examination (cranial nerves, as well as strength and sensation in the arms and legs) and a neuropsychological assessment according to the Glasgow Coma Scale requirements [25]. Those patients with additional non-CNS disorders or under current medication use were excluded from the study.

2.2. Cerebrospinal fluid and serum sample collection

Written informed consent was obtained from each participant or his/her spouses, and the study was approved by the local ethics committee. Upon admission, lumbar puncture was performed between the fourth and fifth lumbar vertebrae with the patient in the lateral decubitus position. The first 1 ml of CSF was discarded and the subsequently obtained CSF collected and aliquotted into polystyrene tubes closed with screw-caps (Sarstedt AG, Nümbrecht, Germany). Peripheral venous blood samples were taken at the time of lumbar puncture. Serum was separated at 4000 *g* after clot-retraction and, such as CSF, stored at −80 °C until further analyses were conducted.

Analysis of the immunoglobulin subclasses and albumin in CSF and serum was performed nephelometrically by a Siemens Healthcare BN2 Nephelometer Analyzer using commercially available kits provided by the company (Siemens Healthcare, Erlangen, Germany). Total protein, glucose and lactate were measured using the Roche Modular Analytics System (Roche, Mannheim, Germany).

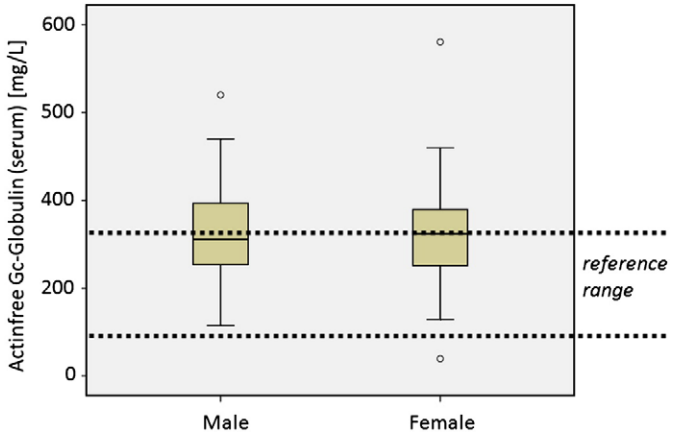

Fig. 1. Comparative demonstration of actinfree Gc-Globulin serum concentrations in male and female patients with multietiological CNS pathologies: No significant difference between the genders was observed, but 41% of the patients with neurodegenerative conditions displayed serum concentrations above the established reference range. Box plots are displayed, where the central line indicates the median per group. The box represents 50% of the values and horizontal lines show minimum and maximum. The dotted line depicts the reference range as provided by the respective manufacturer given in Materials and methods.

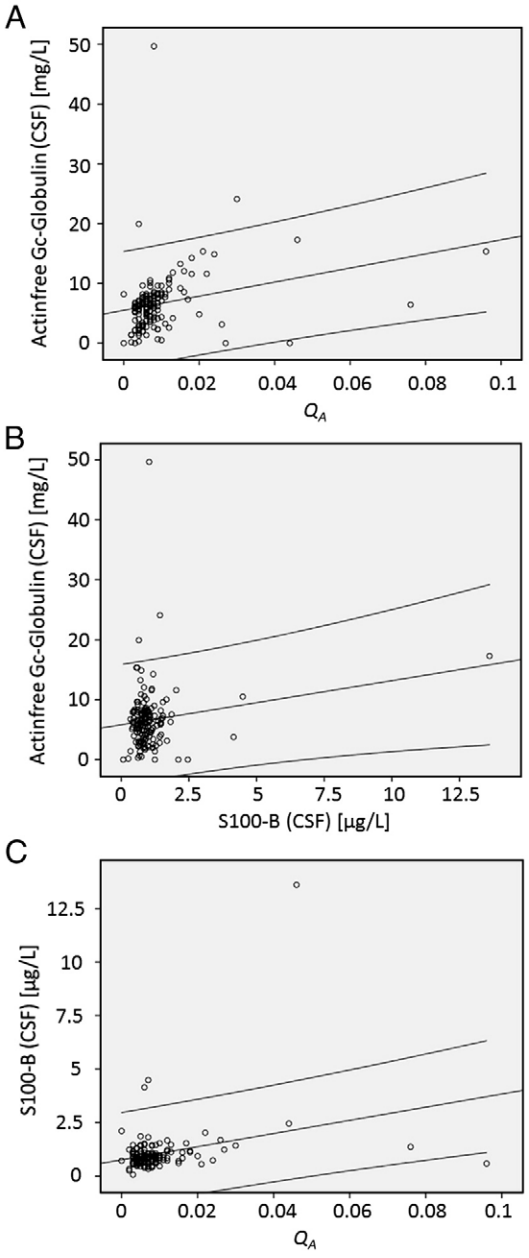

Fig. 2. Association of concentrations of actinfree Gc-Globulin and S100-B in CSF and their relation to the integrity of the blood–brain barrier: Overall, CSF concentrations of actinfree Gc-Globulin (A) and S100-B (C) are positively correlated with the severity of BBB dysfunction [as determined by the ratio of CSF/serum albumin (Q_A)], as well as to each other (B). Shown are range diagrams, regression lines and individual 95% confidence intervals.

2.3. Quantification of S-100B in serum and cerebrospinal fluid

S-100B levels in CSF and serum were analyzed by means of a fully automated electrochemiluminescence assay (ECLIA, Roche Elecsys) with reference values for serum set at <11 µg/L, according to the manufacturer's instructions.

2.4. Quantification of actinfree Gc-Globulin in serum and cerebrospinal fluid

Actinfree Gc-Globulin levels in serum were determined by ELISA (Kit 034, AntibodyShop, Gentofte, Denmark). Reference values for serum ranged from 92 to 332 mg/L, as determined by the manufacturer. Intra-assay (inter-assay) coefficient of variation (CV) ranged from 3.5–3.6% [*n*=6] (3.1–7.9% [*n*=8]).

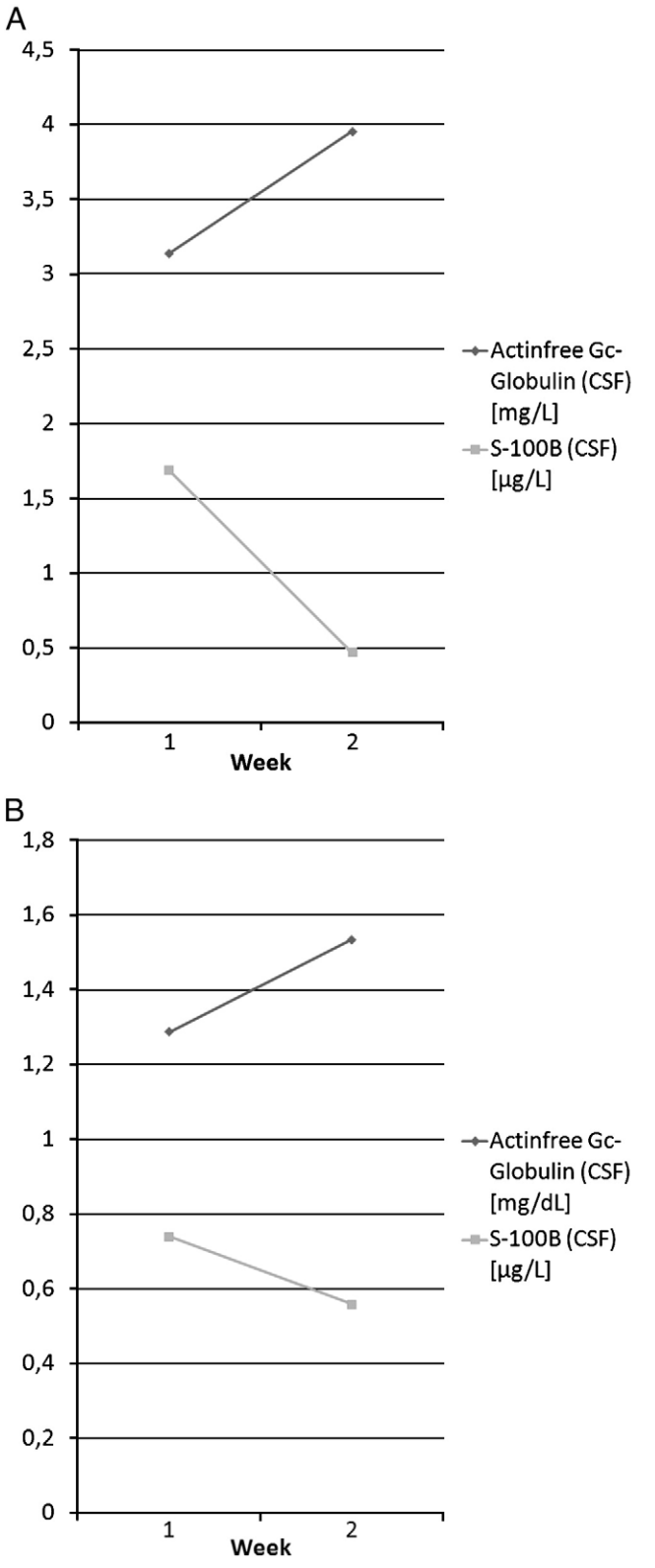

Fig. 3. Development of CSF concentrations of actinfree Gc-Globulin and S100-B over time in two individual patients with CNS pathologies: Shown are measurements in CSF from a patient with pneumococcal meningitis after cranial trauma (A) and from a patient with meningoencephalitis secondary to an abscess in the craniocervical junction with disciitis and osteomyelitis (B). In both patients, CSF was obtained in two separate lumbar punctures upon admission and at day 7 post-admission.

2.5. Assessment of blood–brain barrier function

Due to its reliability as a parameter for the assessment of blood–brain barrier (BBB) function or dysfunction, the median ratio of CSF/serum albumin (Q_A) was 0.009 in all patients. Q_A values below 0.007 were regarded as normal, between 0.007 and 0.01 as a sign of a mild dysfunction, between 0.01 and 0.02 of a moderate and above 0.02 of a severe BBB dysfunction, as previously described by Reiber and Felgenhauer [26].

The ratio of CSF/serum actinfree Gc-Globulin (Q_{Gc}) and albumin (Q_A) was calculated for all paired measurements. In order to determine whether actinfree Gc-Globulin was released intrathecally or whether a passive leakage across a dysfunctional BBB occurred, the actinfree Gc-Globulin-index was calculated as the ratio of Q_{Gc}/Q_A [27].

2.6. Statistical analyses

Statistical analyses were performed using the SPSS 16.0 software (SPSS, Munich, Germany). Due to skewed distributions of most variables, median and range are given. Differences between two groups were assessed by Mann–Whitney-*U*-test or, between more than two groups, by Kruskal–Wallis-ANOVA. Comparisons between subgroups are illustrated with parallel box plot graphics. The boundaries of the box are Tukey's hinges. The median is identified by a line inside the box. The length of the box is the interquartile range (IQR) computed from Tukey's hinges. Values more than three IQRs from the end of a box are labeled as extreme, denoted with an asterisk (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (o). Associations between parameters were calculated by multiple regression analysis which is presented as range diagram with regression line and individual 95% confidence interval. Values of $p<0.05$ were considered statistically significant.

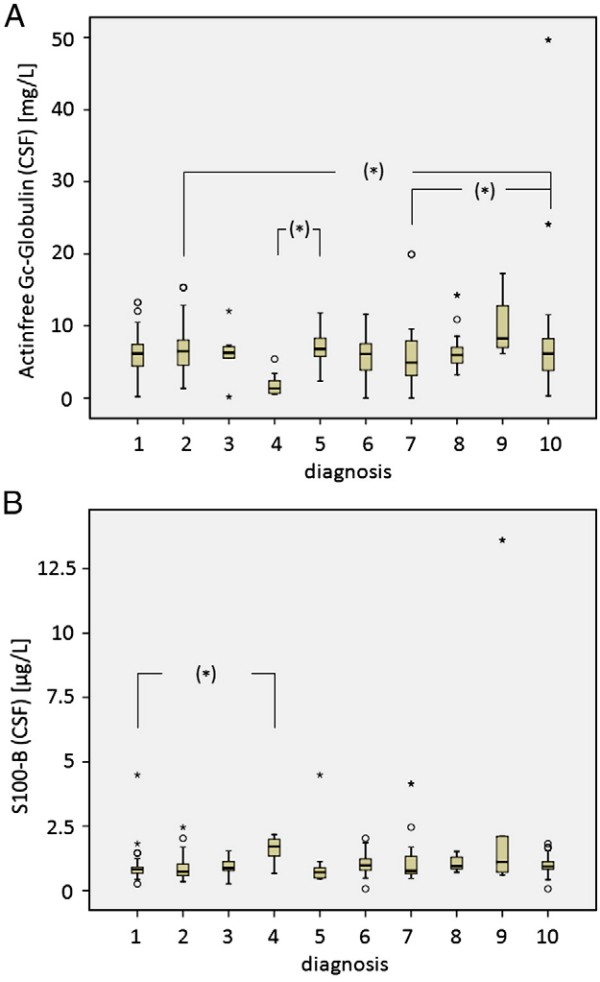

Fig. 4. Actinfree Gc-Globulin (A) and S100-B (B) CSF concentrations in patients with neurodegenerative conditions plotted against etiology. *Diagnosis code:* 1 = Cerebrovascular; 2 = Infectious (viral/bacterial); 3 = Blockade of CSF circulation (hydrocephalus); 4 = CNS trauma; 5 = Autoimmune CNS disease (e.g. MS, Guillain-Barré); 6 = Epilepsy; 7 = Psychiatric disease (e.g. schizophrenia, bipolar disorder, endogenous depression); 8 = Pre-senile dementia (e.g. M. Alzheimer, M. Parkinson, M. Pick); 9 = CNS tumors; 10 = Polyneuropathy (multietiological). Injuries associated with highest S100-B concentrations (in particular CNS trauma [4]) simultaneously display lowest actinfree Gc-Globulin levels in CSF. (*): $p<0.05$.

3. Results

3.1. Actinfree Gc-Globulin and S100-B levels in cerebrospinal fluid and serum of patients with multietiological CNS pathologies

Actinfree Gc-Globulin was measured in paired CSF and serum samples of 155 patients with CNS pathology for a maximum of 14 days post admission.

Upon admission, serum levels of actinfree Gc-Globulin were above cut-off limit in 64 patients and below cut-off limit in 1 patient (mean 323 mg/L, range 39–761 mg/L) (Fig. 1). No difference between the genders was detectable. The mean of all actinfree Gc-Globulin concentrations in the CSF at this time point displayed a range between 0.17 and 49.6 mg/L (mean 6.8 mg/L).

Elevated serum S100-B levels were found in over 11% of the samples analyzed (17 patients) with a range of 0.01 to 0.5 μg/L (mean 0.06 μg/L). In CSF, the mean S100-B level was 1.05 μg/L (range 0.03–4.5 μg/L).

No correlation between obtained Glasgow Coma Scale scores and CSF levels of Gc-Globulin or S-100B was observed. However, it has to be considered that 139 out of 155 patients (i.e. 90%) had scores <10, thus complicating the assessment of a statistical association between these parameters.

3.2. Relationship of actinfree Gc-Globulin in cerebrospinal fluid and disturbance of the blood–brain barrier

The Q_A was calculated in order to assess the BBB function. 76 patients (49%) showed a normal BBB integrity over the entire time course (Q_A<0.007). A mild dysfunction was detected in 43 patients (28%; Q_A=0.007–0.01), a moderate in 24 (15%; Q_A=0.01–0.02) and a severe BBB dysfunction in 12 patients (8%; Q_A>0.02). In 6 patients with disintegrated BBB, a restoration to normal BBB function occurred between 1 and 7 days after admission, whereas in the other patients the respective BBB dysfunction persisted for >7 days. A strong correlation was found between CSF-actinfree Gc-Globulin levels and BBB dysfunction (Q_A>0.02, p=0.006) (Fig. 2A).

3.3. Relationship of actinfree Gc-Globulin and S-100B levels in CSF

The neuroprotein S-100B released into the CSF and circulation is suggested to be a reliable marker for primary brain damage [28,29]. Investigation for an association of actinfree Gc-Globulin and S100-B levels showed a positive correlation of both parameters in CSF (r=0.17, p=0.041) (Fig. 2B). However, CSF levels of S100-B also positively correlated with the extent of BBB dysfunction (r=0.3, p<0.001) (Fig. 2C), initially suggesting a BBB leakage as cause for increased CSF levels of actinfree Gc-Globulin during CNS injury.

3.4. Dependency of intrathecal synthesis of Gc-Globulin on the severity of blood–brain barrier dysfunction

We next aimed to determine whether actinfree Gc-Globulin detected in CSF results from BBB leakage or intrathecal release. Q_{Gc}/Q_A was calculated to identify higher intrathecal or peripheral concentrations of actinfree Gc-Globulin. Q_{Gc}/Q_A>1 indicates that actinfree Gc-Globulin concentrations in CSF exceed serum concentrations. Patients (n=12) with a severe BBB dysfunction presented a higher percentage of Q_{Gc}/Q_A>1 values (42%) compared to patients (n=67) with mild to moderate dysfunction (10%; p<0.0001) and to patients (n=76) with normal BBB function (5%; p<0.0001).

3.5. Time dependent course of actinfree Gc-Globulin and S-100B levels in cerebrospinal fluid of selected patients with central nervous system injury

For six patients two consecutive measurements of actinfree Gc-Globulin and S100-B levels in CSF were obtained, which do not allow to define the time course for these proteins in CSF but which provide a tendency for the dynamic distribution pattern of actinfree Gc-Globulin in the course of CNS injury. Representative data of two patients are shown in Fig. 3A and B, demonstrating the inverse trend of S100-B and actinfree Gc-Globulin CSF concentrations. These data are supported by the comparison of CSF S100-B and actinfree Gc-Globulin among the various aetiologies of CNS disease, showing that those injuries associated with highest S100-B concentrations (e.g. trauma) simultaneously display lowest actinfree Gc-Globulin levels (Fig. 4A and B).

4. Discussion

Several studies have mentioned the presence of Gc-Globulin in CSF of patients with certain CNS pathologies such as Alzheimer and Parkinson diseases [30,31], HIV-1 associated dementia (HAD) [32], lumbar disk herniation [33], or schizophrenia [34]. But despite such phenomenological observations, a pathogenetic role of Gc-Globulin in these conditions is not yet understood.

Katikaneni et al. found that Gc-Globulin levels in the CSF were significantly increased in infants less than 2 months of age with a significant inverse correlation between the age of the patients and CSF Gc-Globulin levels, thus suggesting a possible involvement of this protein in the immunological functions of mononuclear cells and in the increased risk of CNS infections in early infancy [35]. This suggestion may initially be questioned, as later *in vitro* studies demonstrated that particularly the cell-associated form of Gc-Globulin also exerts immunostimulatory functions through enhancing complement factor 5a (C5a)-dependent chemotactic activity, primarily on monocytes and neutrophils (7, 8, 11, 12) through a binding region between amino acid residues 126 and 175. This common sequence is identical among the 3 major isoforms of Gc-Globulin (Gc-1F, Gc-1S, and Gc-2) (6). However recently, we gave evidence that C5a dependent effects are not promoted by increased presence of actinfree Gc-Globulin in severe inflammatory conditions *in vivo*, which again supports the discussion by Katikaneni et al.

In the present study, serum concentrations of actinfree Gc-Globulin were above cut-off limit in 41% of all patients which suggests enhanced hepatic synthesis also in the course of CNS damage. As CSF levels of S100-B, an established marker for primary brain damage [28,29], and actinfree Gc-Globulin correlate but S100-B values are also positively associated to the degree of BBB dysfunction, our data propose that there is no active recruitment of actinfree Gc-Globulin to the site of CNS injury, but that increased CSF levels rather result from BBB leakage. However, even though the major fraction of actinfree Gc-Globulin in CSF seems to be the result of increased extraneuronal synthesis, our data also suggest an increased contribution of intrathecal synthesis of Gc-Globulin in patients with severe CNS injury. This finding, in part, is confirmed by a previous report, suggesting *in situ* synthesis of Gc-Globulin in neurons of the developing brain, and that there may be an important difference in the structure and/or processing of this protein in the CNS, reflecting a function different from hepatic derived Gc-Globulin [36].

The representative data of two patients as shown in Fig. 3 demonstrate an inverse behaviour of actinfree Gc-Globulin and S100-B CSF concentrations, suggesting a consumption of the actin scavenger capacity of Gc-Globulin in times of increased neuronal damage. This finding conforms to previous reports on the increasing presence of cytoskeletal proteins, such as actin, in the CSF of patients with CNS injury and progressive neurodegeneration [6]. This presumption is furthermore supported by the fact that conditions of severe neuronal damage (e.g. CNS trauma), displaying highest S100-B concentrations, are simultaneously accompanied by lowest actinfree Gc-Globulin levels (i.e. residual actin binding capacity of Gc-Globulin).

In summary, confirming the well established role of actinfree Gc-Globulin as actin scavenger in the circulation [10–15], our data propose

a function also in the clearance of actin filaments from CSF of patients with neuronal damage. However, active recruitment of hepatic derived actinfree Gc-Globulin to the site of CNS injury is not observed. Much more, BBB leakage enables extraneuronally synthesized Gc-Globulin to extent its scavenger capacity for actin also to the subarachnoidal space. Furthermore, intrathecal synthesis of actinfree Gc-Globulin seems to be increased in patients with severe neurodegeneration.

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