EXPERIMENTAL STUDY

Follistatin (FS) in human cerebrospinal fluid and regulation of FS expression in a mouse model of meningitis

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Abstract

Objective: Follistatin (FS) is the specific binding protein of activin and expression of both factors is regulated by inflammatory agents. Therefore, FS concentrations were determined in cerebrospinal fluid (CSF) of patients with bacterial and viral meningitis or multiple sclerosis (MS), as well as in the CSF of patients without meningial inflammation or autoimmune diseases. Furthermore, a mouse pneumococcal meningitis model was used to localise the cellular sources of FS in brains of normal and meningitic mice.

Methods: FS concentrations in CSF were determined by ELISA; FS in mice was localised by in situ hybridisation and immunohistochemistry.

Results: FS concentrations were ≥0.4 μg/l in 22 of 66 CSF samples of meningitis patients versus 2 of 27 CSF samples from patients with multiple sclerosis (P < 0.05) and 2 of 41 CSF specimen from patients without neuroinflammatory diseases (P < 0.01). In the CSF of patients with meningitis, the concentration of FS was correlated with total protein (P < 0.005) and lactate concentrations (P < 0.05), but not with leukocyte counts, interval between onset of disease and CSF analysis, or clinical outcome. The CSF-to-serum ratios of FS and albumin also correlated significantly (P < 0.0005). In some patients with meningitis the CSF-to-serum ratios suggested that the elevated FS in CSF did not originate from serum alone. FS was localised in mice brains to neurones of the hippocampus, dentate gyrus, neocortex, and to the choroid plexus. Analyses of brains and other organs from uninfected and infected animals sacrificed 6–36 h after infection did not reveal any obvious differences in the distribution and intensity of FS mRNA and protein expression.

Conclusions: The concentration of FS in humans is elevated during meningitis. In some patients the increase is caused by a release of FS from brain into CSF. Data from the mouse meningitis model suggest that increased CSF concentrations of FS in meningitis appear not to be accompanied by an elevated number of cells containing FS mRNA or protein in the brain.

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Introduction

Activin is an almost ubiquitous cytokine (1, 2) that effects growth, differentiation and function of multiple tissues and organs including pituitary hormone secretion (3–5) and neural differentiation (6–8). FS specifically binds to activin (9). As a result, FS neutralises activin actions by preventing the interaction of the cytokine with its type II receptors (10) and by facilitating its lysosomal degradation (11).

Recently, a neuroprotective effect of i.c.v. administered activin A on hippocampal and striatal neurones in hypoxic-ischemic brain injuries in rats has been reported (12); activin A administered i.c.v. also increased luteinising hormone concentrations, but not follicle-stimulating hormone serum levels in adult male rats (13). Furthermore, intrastriatal infusion of activin A has a protective effect on striatal cholinergic interneurone populations in quinolinic acid-induced degeneration (14). During focal brain injury, activin βA mRNA expression is increased in hippocampal neurones (15). Shoji et al. discovered an activin type II receptor specific for neurones (16). In mice of all ages, the highest levels of activin A binding were observed throughout the brain, spinal cord, and trigeminal and spinal ganglia, whereas in activin receptor II-deficient mice, activin A binding was absent in neural tissues (17). FS, too, is widely expressed in the brain (18). This
suggests that the balance of FS and activin, rather than activin alone, is important for the development and function of neuronal tissues.

FS can be measured in human and animal serum; its source, however, has yet to be determined (19–35). FS expression in vitro (36) and in vivo (36–38) is increased upon challenge with bacteria, and FS and activin might play roles in acute phase response and infection (39). Although much is known about FS concentrations in serum, amnion and follicular fluid, hardly anything is known about FS protein levels in CSF. Using Western blotting, we demonstrated that FS protein is present in CSF and that the choroid plexus expresses FS mRNA (36). In the present study, we investigated whether FS concentrations in CSF change during infectious and autoimmune diseases by analysing the FS concentration in CSF samples from humans with viral and bacterial meningitis, multiple sclerosis and without inflammatory diseases. A mouse meningitis model was used to localise the cellular sources of FS mRNA and protein in meningitic brains and to determine whether bacteria increase the number of FS mRNA- and protein-expressing cells in the brain. Meningitic mice developed a sepsis during the disease, and therefore, other organs were also examined.

Materials and methods

Sample collection

CSF and blood were collected according to clinical necessities from male and female patients of different ages with bacterial or viral meningitis (66 samples from 45 patients), multiple sclerosis (27 samples from 27 patients) and from patients who presented with a variety of symptoms, but who had no signs of an autoimmune or infectious disease (41 samples from 41 patients). After completion of the clinical routine analyses, FS was measured in the remaining CSF and serum; no extra CSF or blood samples were drawn from the patients for the purpose of this study. The samples were stored frozen at -20 °C until assay. The groups did not differ significantly with respect to age and sex.

ELISA

FS concentrations (free FS plus activin-bound FS) were determined by an enzyme immunoassay described and validated previously. Cross-reactivities with recombinant human activin A and bovine inhibin were <0.1%. The intra- and interassay coefficients of variation were <10%, and the recovery rate was 90% in human serum. The assay cross reacts fully with human recombinant FS 288 and detects activin-bound and unbound FS (40). In the ELISA, recombinant human FS 315, kindly provided by Dr Y Eto (Ajinomoto Central Research Laboratory, Kawasaki, Japan), was used as the FS standard. Total protein and albumin were measured by nephelometry (Dosacat, Dosatech, Munich, Germany). CSF proteins were precipitated with trichloroacetic acid (final concentration of 2.45 M) and albumin with a specific antibody respectively. Lactate concentrations were determined enzymatically (Greiner Biochemica, Flacht, Germany). CSF leukocytes were counted with a Fuchs-Rosenthal chamber by adding 10 μl Garrelts’ dye (5 ml acetic acid + 32 ml Ziehl carbolfuchsin + 63 ml H₂O) to 90 μl CSF. The CSF-to-serum ratios of FS [Q(FS)] and albumin [Q(Alb)] were calculated.

Western blotting

CSF containing 60 μg of protein was lyophilised to dry powder at room temperature and reconstituted in 50 μl SDS-PAGE sample buffer (0.36 M bistris, pH 7.7, 0.16 M bicin, 15% saccharose, 2% SDS, 2.5% β-mercaptoethanol). Samples were electrophoresed, blotted and immunostained as described earlier (36, 41), except that the dilution of the first antibody was 1:4000. The porcine FS standard and the polyclonal antibody were generous gifts from Dr Hiromu Sugino, Institute for Enzyme Research, University of Tokushima, Japan (42).

Mouse meningitis model

C57Bl mice (2–3 months old) with free access to food and drinking water were anaesthetised with ketamine 100 mg/kg and xylazine 20 mg/kg and infected by injecting approximately 10 000 colony-forming units (CFU) of a Streptococcus pneumoniae type 3 strain (MIC/MBC: 0.03/0.06 g/ml for ceftriaxone) into the right forebrain (43). They were not treated with antibacterials. The health status of the mice was assessed by weighing, by a clinical score (no apparent behavioural abnormality: 0; moderate lethargy: 1; severe lethargy: 2; unable to walk: 3) and by the tightrope test (44). Animals were anaesthetised 6, 12, 18, 24, and 36 h after infection, and a suboccipital puncture was performed. All animals were killed by decapitation and blood was sampled. Then, the whole brain, spleen and all other tissues examined were removed. The cerebellum and one half of the spleen were homogenised in saline. Serial dilutions of blood, cerebellum and spleen homogenates were plated on blood agar plates. Pooled CSF from three animals each was used for the determination of leukocyte count and bacterial titers. Tissues for in situ hybridisation and immunohistochemistry were fixed in 4% formalin for 48 h and then embedded in paraffin. All animal experiments were approved by the District Government Braunschweig, Germany.

cRNA probes

A partial FS clone was isolated from a mouse kidney cDNA library in lambda ZAP (Stratagene, Heidelberg,
Germany) with a heterologous rat FS probe (45). A fragment of this clone containing bp 407–1020 was subcloned in pGEM4Z. Antisense cRNA was transcribed from EcoRI linearised plasmid with T7 polymerase, and sense cRNA was transcribed from XbaI linearised plasmid with Sp6 polymerase.

In situ hybridisation and immunohistochemistry

Immunohistochemistry and in situ hybridisation were performed on 1–2 μm thick sections of paraffin-embedded mice tissues mounted on poly-L-lysine-coated glass slides. The sections were deparaffinised and hydrated. For immunohistochemistry sections were boiled five times in a microwave for 3 min in citrate buffer (10 mM citric acid monohydrate, pH 6.0). Sections were then incubated with the polyclonal rabbit antibody against FS (42) at a dilution of 1:1000 in Tris-buffered saline (TBS; 25 mM Tris and 150 mM NaCl, pH 7.5) for 2 h at room temperature (RT) in a water-saturated atmosphere. Specific binding of mouse FS antigen was tested by Western blotting. The sections were then incubated with mouse anti-rabbit immunoglobulins (diluted 1:50 in TBS; DAKO, Glostrup, Denmark), rabbit anti-mouse immunoglobulins (diluted 1:50 in TBS; DAKO) for 30 min each and alkaline phosphatase anti-alkaline phosphatase (diluted 1:50 in TBS; DAKO) for 1 h at RT. Sections were then incubated in neufuchsin substrate and counter stained blue with Mayer’s hemalum solution (1:1 dilution in water). Specific binding of the FS antibody was visible as dark-pink staining.

For in situ hybridisation, sections were incubated in 4% paraformaldehyde for 20 min at 4 °C. After incubation in 0.2 M HCl for 10 min at RT, sections were incubated in 0.5% acetic anhydride (in 100 mMTris, pH 8) for 10 min at RT with gentle shaking. The sections were then treated with proteinase K (10 μg/ml in TBS containing 2 mM CaCl₂) for 20 min at 37 °C. After washing the sections three times in TBS at RT and once in ice-cold TBS they were dehydrated with ascending ethanol concentrations and dried at 60 °C for 40 min. Hybridisation was performed overnight at 65 °C in a solution containing 2-fold SSC, 50% deionised formamide, 10% dextran sulfate, 0.01% herring sperm DNA, and 0.02% SDS, and either the sense or antisense digoxigenin-labelled cRNA probe specific for mouse FS. Sections were then washed twice in wash buffer I (1-fold SSC containing 0.1% SDS) for 10 min at RT and twice in wash buffer II (0.2-fold SSC containing 0.1% SDS) for 10 min at 60 °C. Before incubation of the sections with the anti-digoxigenin alkaline phosphatase Fab fragments (diluted 1:250 in Boehringer blocking reagent containing 10% FCS and 3% mouse serum) for 2 h at RT, non-specific binding of the Fab fragments was blocked by treating the sections with blocking reagent containing no Fab fragments for 15 min. Then, sections were washed five times in TBS at RT and incubated in NBT/BCIP chromogenic substrate at 4 °C. Finally, sections were counter stained with nuclear fast red (0.1% in 5% aluminum sulfate) resulting in a light-red background and dark-red nuclei.

Data analysis

Differences in serum and CSF FS concentrations between groups were analysed by the two-tailed non-parametric Kruskal–Wallis test followed by
Dunn’s multiple comparison test. To detect correlations between the individual parameters measured in serum or CSF, Spearman’s rank correlation coefficients were determined. For the discrimination of passive diffusion of FS from blood to CSF and FS release from nervous tissue into CSF, Q(FS) and Q(Alb) were calculated. *P* values <0.05 were considered significant.

**Results**

Of the 66 samples from 45 patients with bacterial meningitis, 22 samples had FS CSF concentrations above the detection limit of the assay (≥0.4 µg/l) ranging from 0.42 to 6.88 µg/l; no difference in FS CSF concentration was found between patients with bacterial or viral meningitis. Analysis of the 27 samples from MS patients revealed only two samples (0.44 and 0.66 µg/l) above the detection limit. The same applied to the analyses of the 41 control samples that also identified only two samples (0.41 and 0.42 µg/l) with concentrations above the detection limit. As shown in Fig. 1A, FS concentrations in CSF from patients with meningitis differed significantly from controls (*P*, 0.01) and MS patients (*P* < 0.05). In the CSF of patients with meningitis, the concentration of FS was correlated with total protein (*P* < 0.005) and lactate concentrations (*P* < 0.05), but not with leukocyte counts, interval between disease onset and CSF analysis, or outcome. In contrast, there was no difference between the three groups with respect to the FS serum concentrations (Fig. 1B). The median FS serum values and the 25th and 75th percentile were: for control patients, 4.9 (3.5/7.4) µg/l; for MS patients, 3.8 (2.6/6.8) µg/l; and for patients with meningitis, 4.5 (2.8/6) µg/l respectively. These values were within the normal range of healthy controls (5–13 µg/l) (24, 35, 46).

In CSF samples with detectable FS, Q(Alb) and Q(FS) correlated significantly with *r* = 0.7 (*P* < 0.0005). In all except one (no. 12) of these 22 samples, Q(Alb) was increased above the normal range (≥0.008). Between a Q(Alb) of 0.015 and 0.030 (moderate elevation of Q(Alb)) several CSF/serum pairs with high CSF concentrations and a Q(FS) >0.25 were observed (Fig. 2 and Table 1).

To determine the size of FS in human CSF, Western

![Figure 2](http://www.eje.org/)

*Figure 2* Double logarithmic plot of Q(FS) versus Q(Alb) (*r* = 0.7, *P* < 0.0005). Values with a threefold higher Q(FS) than Q(Alb) are above the estimated line and are shown as ●. The estimated line separates Q(FS) originating from diffusion through the blood–CSF barrier and Q(FS) which can only be explained by an additional release of FS from intracranial sources.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Q(Alb)</th>
<th>Q(FS)</th>
<th>Increase in Q(Alb) (%)</th>
<th>Increase in Q(FS) (%)</th>
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<td>1</td>
<td>0.034</td>
<td>0.1</td>
<td>520</td>
<td>830</td>
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<tr>
<td>2</td>
<td>0.019</td>
<td>0.12</td>
<td>290</td>
<td>1000</td>
</tr>
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<td>3</td>
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<td>0.091</td>
<td>700</td>
<td>760</td>
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<td>4</td>
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<td>0.28</td>
<td>320</td>
<td>2330</td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>22</td>
<td>0.031</td>
<td>0.47</td>
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<td>3920</td>
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blots of CSF from patients with meningitis and controls were performed. Fig. 3 shows that FS can be detected in 60 μg of CSF protein from patients with bacterial meningitis but not in the equivalent amount of CSF from control patients. The result is in accordance with data from an earlier study (36) and revealed a major band of approximately 31 kD in human CSF and in porcine ovarian follicular fluid. Minor bands of sizes similar to those of the porcine FS standard were also detected in some CSF samples.

As no fresh human post-mortem brains were available, we used brain tissue from a mouse meningitis model to localise FS mRNA and protein. Progression of the disease was assessed by leukocyte counts and bacterial titer in the CSF. Leukocyte counts and bacterial titers for the pooled CSF at 6 h were 235/μl and 10^6 CFU/ml respectively, at 12 h 1237/μl and 3.2 x 10^6 CFU/ml respectively, at 18 h 5291/μl and 10^7 CFU/ml respectively, at 24 h 10581/μl and 10^8 CFU/ml respectively, and at 36 h 7200/μl and 10^9 CFU/ml respectively. CSF mRNA and protein were localised by in situ hybridisation (Fig. 4A, D and G) and immunostaining (Fig. 4C, F and I) to the neurones of the hippocampus (Fig. 4A and C), dentate gyrus (Fig. 4A) and neocortex (Fig. 4D and F) as well as to the epithelial cells of choroid plexus (Fig. 4G and I); no specific signals above background were detected in the meninges. No obvious differences in FS mRNA and protein expression were detected between animals with meningitis killed between 6 and 36 h after infection and healthy controls. Furthermore, S1 nuclease analysis of brain RNA derived from meningitic mice killed 36 h after infection and healthy animals also did not reveal any substantial differences in the steady-state levels of FS RNA. As all meningitic mice developed sepsis during the course of the disease, we also examined liver, spleen, lung, heart, muscle, skin, gut, male and female gonads, and kidney with in situ hybridisation and immunohistochemistry. Some diseased animals had stronger stainings in the glomeruli of the kidneys than the respective controls, but, in the other organs, no differences were detected in the intensity and pattern of FS mRNA and protein expression between healthy and diseased animals.

**Discussion**

Molecules in serum cross the blood–brain and blood–CSF barrier via passive diffusion. In addition to diffusion, many molecules are actively transported into or out of the CSF. The contribution of diffusion, facilitated diffusion or energy-dependent active transport to the CSF concentration of a certain molecule depends on the compound studied. The extent by which molecules diffuse through the blood–CSF barrier depends on their chemical and physical abilities (in particular size, shape, lipophilicity, polarity and binding to serum proteins) (47). Provided that the blood–CSF and blood–brain barriers are intact, the CSF/serum concentration ratio of a certain protein spans a narrow range, which is characteristic of the molecule (49). The CSF-to-serum ratio of a molecule [Q(X)] changes if either its clearance from cerebrospinal fluid or its diffusion through the blood–brain barrier or blood–CSF barrier is altered, or if it is released from other central nervous system compartments into the CSF.

Due to differential splicing and different forms of glycosylation, the size of FS varies between 31 and 45 kD (7, 48). In this study, the major form of FS in CSF detected by Western blotting was 31 kD. CSF FS concentrations below detection limit in most controls and MS patients did not allow the calculation of a normal range for Q(FS) from our data, but the smaller sizes of the most frequent FS molecules in CSF in comparison to albumin (66 kD) imply that Q(FS) should be slightly larger than Q(Alb). In adults Q(Alb) increases with age and ranges from 5–8 x 10^{-3}. From Fig. 2 and Table 1 it is apparent that all of the samples, with measurable CSF FS concentration had a higher Q(FS) than Q(Alb).

Albumin is exclusively synthesised in and secreted from the liver. Therefore, the increased Q(Alb) seen in all CSF and serum specimens listed in Table 1 (except in no. 12) indicate a decreased clearance of albumin from the CSF and/or an increased permeability of the blood–CSF barrier in these patients. The increases in Q(Alb) range from 1.2- to 30-fold based on an average Q(Alb) of 6.5 x 10^{-3} in healthy individuals. The (CSF/serum) for molecules of the average size of FS, in the presence of an intact blood–CSF barrier and a normal
CSF flow, is approximately $7-12 \times 10^{-3}$ (49). Based on a $Q(\text{FS})$ of $12 \times 10^{-3}$, the increases in $Q(\text{FS})$ ranged from 4.2- to 105-fold (Table 1).

The relations between $Q(\text{Alb})$ and $Q(\text{IgG})$, $Q(\text{Alb})$ and $Q(\text{IgA})$ and $Q(\text{Alb})$ and $Q(\text{IgM})$ are hyperbolic functions. Within a $Q(\text{Alb})$ range of $3 \times 10^{-3}$ to $100 \times 10^{-2}$, these functions have almost linear slopes when plotted in a double logarithmic manner. In healthy individuals, the FS concentrations in CSF are below the detection limit of the assay. This prevented the calculation of a function describing the relation of $Q(\text{Alb})$ and $Q(\text{FS})$. The line in Fig. 2 was calculated on the assumption that a threefold higher increase of $Q(\text{FS})$ compared with $Q(\text{Alb})$ cannot be explained solely by an increased diffusion or/and a reduced clearance of FS from the CSF. The validity of this assumption is supported by the fact that the $Q(\text{FS})$ of patient no. 5 (1.26 in Table 1) cannot be explained without the assumption of an intracranial release of FS (FS CSF concentration $>$FS serum concentration). This value is still on the line in Fig. 2, intended to discriminate passive diffusion and additional release from other CNS compartments. The plot of $Q(\text{FS})$ versus $Q(\text{Alb})$ revealed eight values which are above the estimated discrimination line for $Q(\text{FS})$ (Fig. 2). In view of these data, it can be concluded that FS concentrations in CSF increase in response to inflammation, similar to the results seen in serum from humans with sepsis (29) and in animals after injection of endotoxin (37) or yeast (38).

As we did not find any significant correlation between FS concentrations and leukocyte counts in the CSF samples, it is unlikely that the increased FS concentrations in CSF from patients with meningitis originate from leukocytes migrating from the blood to CSF. We also did not find any correlation of FS concentrations in CSF and the clinical outcome, making increased FS concentrations as a consequence of tissue destruction within the CNS unlikely. As CSF lactate levels rise...
during the critical phase of bacterial meningitis as a consequence of anaerobic glycolysis in the intracranial compartments. The correlation of lactate and FS concentrations in CSF underscores a role of FS in the response of the CNS to infection. Similar to our finding of increased serum levels of FS in patients with septicaemia, increased CSF concentrations of FS were only found in some of the samples analysed from patients with meningitis. This is probably due to the transient effect of FS stimulation by proinflammatory agents, as seen in animal (37, 38) and human serum (29) or during in vitro experiments (36).

To determine the origin of elevated CSF concentrations, FS mRNA and protein was studied in healthy and meningitic mice by in situ hybridisation and immuno-histochemistry. Induction of FS mRNA (36) and protein (29, 38) by bacterial compounds in humans and animals and increased FS CSF values in some patients with meningitis stimulated us to search for FS mRNA and/or FS protein in brain cells of meningitic mice. Surprisingly, although the localisation of FS mRNA and protein in mouse brain was in accordance with data from rat (18) and human tissues (50), we did not detect substantial differences in FS mRNA and protein expression between mice with meningitis and controls. Therefore, proinflammatory agents seem not to increase the number of FS-synthesising cells in brain tissue, but rather elevate the secretion and/or production of FS protein from FS positive cells. The same seems to be true for other organs of mice suffering from sepsis, and the kidney might be the only exception.

In summary, FS concentrations in CSF are elevated in samples from patients with viral and bacterial meningitis, but not in samples from MS patients and control patients without inflammatory or autoimmune diseases. Our data confirm an involvement of FS in the inflammatory response and demonstrate that this effect is not restricted to the blood but also occurs in CSF. In some patients the increase in FS CSF concentrations can only be explained by a release of FS from other intracranial compartments into the CSF in response to the infection. Results from animal experiments suggest that an increase of the number of FS-synthesising cells within the CNS during meningitis is rather unlikely. The role of FS in infection and inflammation of the CNS might be the maintenance of a well-balanced steady-state equilibrium of FS and activin to control the effects of activin in brain.

Acknowledgements

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