Review

Transferrin isoforms in cerebrospinal fluid and their relation to neurological diseases

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Abstract: Iron plays many important roles in the brain, including involvement in myelination, neurotransmission and electron transfer in the respiratory chain. Transferrin (Tf), an iron transporter, is mainly biosynthesized in the liver, but can also be biosynthesized in the brain; i.e., by oligodendrocytes and the choroid plexus, a cerebrospinal fluid (CSF) producing tissue. The CSF contains two Tf isoforms, brain-type Tf and serum-type Tf, which differ in their glycan structures. Brain-type Tf is uniquely glycosylated with biantennary asialo- and agalacto-complex type N-glycans that carry bisecting β1,4-GlcNAc and core α1,6-Fuc. The glycans of serum-type Tf in the CSF are similar to those of Tf in serum. Biochemical analyses reveal that the apparent molecular size of brain-type Tf is smaller than that of serum-type Tf, and that hydrophobic patches are exposed on brain-type Tf as demonstrated by hydrophobic probe binding studies. We found that brain-type Tf levels were decreased in idiopathic normal pressure hydrocephalus, in which CSF production is suspected to decrease, while brain-type Tf increased in spontaneous intracranial hypotension, in which CSF production is suspected to increase. These results suggest that brain-type Tf could be a biomarker of altered CSF production.

Keywords: biomarker, cerebrospinal fluid, transferrin, lipocalin-type prostaglandin D synthase, intracranial hypotension syndrome, idiopathic normal pressure hydrocephalus

Introduction

Iron is an essential nutrient for physiological processes throughout the body, including the brain. Oligodendrocytes require iron for myelination and store large amounts of ferritin-bound iron. In contrast, astrocytes retain less iron.1) As a multi-purpose nutrient, iron is involved in oxygen transport, electron transport and many other fundamental physiological processes.2) However, free iron results in cellular toxicity by generating harmful reactive oxygen species (ROS).3) Iron, therefore, needs to bind to specific carrier proteins such as transferrin (Tf) in the extracellular fluid and blood.4)5) Tf is a single polypeptide chain with 679 amino acid residues, consisting of two homologous N- and C-terminal domains.6) Each domain has a metal binding pocket with high affinities for ferric iron, which are crucial for preventing ROS generation and subsequent cell damage.

Central nervous system (CNS) iron is mainly derived from blood Tf.7) However, the CNS is separated from the systemic circulation by the blood-brain barrier (BBB), which is comprised of endothelial cells, basement membrane, pericytes and astrocytic processes. Blood Tf binds to transferrin receptor 1 (TfR1) on endothelial cells lining blood capillaries and the binding complex is internalized...
into endosomes (Fig. 1). After internalization of the Tf/TfR1 complex, the iron dissociates from Tf in an acidic endosomal compartment. The iron-free transferrin (apotransferrin) together with TfR1 is then transported back to the plasma membrane where it is released into the blood to participate in further rounds of iron mobilization and delivery. Ferric iron (Fe$^{3+}$) released from Tf is reduced to ferrous ion (Fe$^{2+}$) by endosomal reductases and then transported in the cytosol by divalent metal transporter 1 (DMT1). It is then exported to the abluminal side by ferroportin. The exported Fe$^{2+}$ is oxidized to Fe$^{3+}$ by GPI-anchored ceruloplasmin on astrocytic process. Fe$^{3+}$ binds to Tf in the interstitial fluid (Fe$^{3+}$/Tf), which is taken up by neuron. In the interstitial fluid, some Fe$^{2+}$ binds to ATP, citrate, and ascorbic acid (non-transferrin-bound iron; NTBI).

Neurons express high levels of TfR1, which binds the Tf/Fe$^{3+}$ complex to be internalized. After internalization, the iron is released and either stored as ferritin-bound iron or passed into the mitochondria, where iron is incorporated into haem and iron-sulphur clusters. There are two possible pathways that Tf can follow after the internalization; one is "recycling" in the brain interstitial fluid, as is the case for blood Tf, and the other is degradation in neurons. It remains to be clarified, however, which pathway is dominant in the neuron. In contrast to neurons, oligodendrocytes and astrocytes take up iron as non-transferrin-bound iron (NTBI), in which Fe$^{2+}$ binds to ATP, citric acid and ascorbic acid (Fig. 1).

It was demonstrated that Tf is biosynthesized by neural cells such as oligodendrocytes and CSF-producing epithelial cells in the choroid plexus. Blay et al. reported that Tf mRNA is expressed in choroid plexus epithelium, while De Arriba Zerpa et al. reported that Tf mRNA in rat primary cultured oligodendrocytes is spliced and lacks the N-terminal signal sequence. These findings suggest that Tf proteins produced by oligodendrocytes reside in the cytosol and are not secreted.
Transferrin and its glycan isoform in cerebrospinal fluid (CSF)

When the protein compositions of human serum and cerebrospinal fluid (CSF) are compared using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining, the major CSF protein bands are similar to those in serum except for the added presence of lipocalin-type prostaglandin D synthase (L-PGDS) and transthyretin (TTR) (Fig. 2A). The result is consistent with a previous report showing that 80% of CSF proteins are derived from the serum. The specimens were also examined by Western blot analysis using antibodies against serum glycoproteins with a view to detecting CSF-specific isoforms. Indeed, Western blot analysis using anti-Tf antibody detected two isoforms in CSF: one with a similar migration position to serum Tf and the other with a faster migration position (Fig. 2B). This latter isoform is named “brain-type” Tf, also designated as asialo-Tf or Tf-1, while the former isoform is named serum-type Tf.
Tf sialo-Tf or Tf-2.\textsuperscript{11–13} The concentrations of brain-type and serum-type Tf, quantified by chromatography (Fig. 3A).\textsuperscript{18} Fluorescence correlation spectrometry analysis revealed that the hydrodynamic radius of brain-type Tf was smaller than of serum Tf, which was consistent with the size-exclusion chromatography data. These results suggested that the conformation of brain-type Tf is changed by a unique glycan. The isoform was, therefore, subjected to X-ray crystallographic analysis; however, the conformation of the polypeptide main chain (data not shown) was very similar to that of a previously reported Tf structure,\textsuperscript{19} PDB code 3V83. We subsequently examined the surface properties of isoforms using the fluorescent probe ANS (1-anilino-8-naphthalenesulfonate), which has been widely used to monitor transitions in proteins owing to its affinity for partially exposed hydrophobic regions.\textsuperscript{20} Addition of ANS to brain-type Tf induced a marked enhancement of fluorescence,\textsuperscript{18} with a maximum at 492 nm. In contrast, ANS shows only weak fluorescence with serum Tf at 504 nm. ANS alone shows weak fluorescence at 508 nm. The strong, red-shifted fluorescence of ANS in the presence of brain-type Tf suggests that it binds to an exposed hydrophobic surface or pocket in this Tf isoform.

Next, we examined functional differences in Tf isoforms. The most evident function of Tf is its high affinity binding to ferric iron. Equilibrium dialysis analysis using \textsuperscript{59}Fe revealed that the isoforms did not show any apparent difference in iron binding affinity (unpublished data). The isoforms were further examined for their binding to Tfr1, which was stably expressed on CHO cells. Again, the isoforms did not show any difference in binding affinity for the receptor (unpublished data). Although Tf glycans influence physicochemical properties such as the hydration volume and exposure of hydrophobic patches, they did not affect any of the functions we examined. Further analyses are required to identify potential functional differences between the isoforms.

Histochemistry using anti-Tf antibody and lectins

To examine the origin of brain-type Tf, we analyzed the CSF of patients with hydranencephaly, a congenital malformation of the brain. In hydranencephaly, the cerebral hemispheres are absent due to bilateral occlusion of the internal carotid arteries, whereas the midbrain, cerebellum and other brain structures nourished by the vertebral artery may remain.\textsuperscript{21,22} Brain-type Tf was decreased markedly in the CSF of hydranencephaly patients, indicating...
that brain-type Tf is derived from the cerebrum (Fig. 2C).23)

We next examined the localization of Tf in the human cerebrum. Immunohistochemistry using anti-Tf antibodies revealed that the most intense signal was detected in the choroid plexus epithelium (Fig. 4A, B). Perls’ staining to detect iron also revealed strong signals in the choroid plexus epithelium (Fig. 4C), suggesting that holo-Tf (iron-saturated form of Tf) is formed in the epithelium and secreted into the CSF. To analyze glycan(s) expressed by the epithelium, the choroid plexus was first co-stained with PVL lectin and Tf-antibody, with the latter staining portions of the choroid plexus epithelium, interstitial tissues and capillary blood clots (Fig. 4E, left panel). High magnification imaging showed preferential staining of basolateral aspect of the epithelium, with minimal staining of apical aspect (Fig. 4E, right panel). The basolateral signals partially overlapped with those of PVL lectin (Fig. 4G, right panel). Other areas were minimally stained with PVL lectin, suggesting that GlcNAc-terminated glycoproteins are localized specifically in the epithelium (Fig. 4F). Next, the choroid plexus was co-stained with SSA lectin and anti-Tf antibody. SSA strongly stained some interstitial tissues and blood clots (Fig. 4J, left panel), with SSA staining.

Fig. 3. Physicochemical analysis of Tf isoforms. Estimation of molecular size of serum- and brain-type Tf by size-exclusion chromatography (A). The retention time of standard proteins (aldolase, 158 kDa, 23.4 min; conalbumin, 75 kDa, 25.2 min; ovalbumin, 43 kDa, 26.4 min) is indicated with black triangles. Fluorescence correlation spectroscopy (FCS) analysis of Tf isoforms (B). FCS shows Stokes–Einstein hydrodynamic radius of serum-type and brain-type Tf. Fluorescence spectra of 1-anilino-8-naphthalene sulfonate (ANS) binding assay for Tf isoforms (C). ANS alone (black solid line), presence of brain-type Tf (red line) or serum Tf (black dotted line). Protein and ANS concentrations were 2 and 140 µM, respectively. The excitation wavelength was set at 390 nm.
substantially overlapping with strong anti-Tf antibody positivity (Fig. 4K, left panel). In contrast, the majority of SSA staining on basolateral portions of the epithelium did not overlap that of Tf antibody (Fig. 4K, right panel). These results suggest that the epithelium specifically expresses GlcNAc-terminated glycoproteins in choroid plexus tissue and possibly produces brain-type Tf.23)

Changes in brain-type transferrin in neurological disease

Idiopathic normal pressure hydrocephalus (iNPH). Patients with idiopathic normal pressure hydrocephalus (iNPH) have excess intracranial CSF, which compresses the brain parenchyma. Resulting in cognitive impairment, gait disturbance, and urinary incontinence among the major symptoms. In iNPH, excess CSF may decrease further CSF production.25) At least two CSF biomarkers have been identified for iNPH. These include L-PGDS, which is mainly produced by the leptomeninges, arachnoid membrane, and choroid plexus,26) while soluble amyloid precursor protein (sAPP) is secreted by neurons.27) L-PGDS and sAPP are decreased in the CSF of iNPH patients,28),29) which fits with a previous report by us.12) Brain-type Tf levels are decreased in the CSF of iNPH patients (Fig. 5A, B). The concentration of brain-type Tf in the CSF was quantified by chromatocanning as shown in Fig. 5C. Patients with suspected iNPH can be further diagnosed by lumbar puncture or continuous lumbar drainage, in which excess CSF is temporally removed as a means to improvement symptoms. In continuous lumbar drainage, the patient undergoes continuous withdrawal of CSF for 6 days. Compared to day 0, the Tf isoform concentration increased from day 1–3 (126 ± 11%, not significant) and more conclusively from day 4–6 (143 ± 17%, p < 0.01) (n = 4), indicating that brain-type Tf production began to increase within a week of drainage onset (Fig. 5D). Data on serum-type Tf is not included in Fig. 5 because the lumbar drainage procedure may result in contamination with blood Tf, the concentration of which is 40–50-fold higher than that of CSF.

The long-term recovery of brain-type Tf was analyzed after shunt surgery for hydrocephalus. The isoform is markedly increased during post-operative
months 1–3 and then gradually declines from months 6–24, suggesting that brain-type Tf is an indicator for the recovery of CSF production (Fig. 5E). In contrast, serum-type Tf levels showed subtle change in the CSF of iNPH patients after shunt surgery (data not shown). Serum-type Tf influx from the blood to the CSF probably occurs due to passive diffusion, meaning that its concentration before and after surgery does not change.

We found that neurological clinical grading scales in response to shunt surgery improved after 11.8 ± 7.7 months. When correlations between cognitive scales and CSF biomarkers were analyzed at various time points, brain-type Tf levels before shunt surgery showed low ($r = 0.351$, $p = 0.042$) and moderate ($r = 0.527$, $p = 0.002$) correlations with Mini-Mental State Examination (MMSE) and Frontal Assessment Battery (FAB) scores, respectively (Fig. 6A, B). At 3 months post-operative, the increase of brain-type Tf correlated strongly with MMSE scores ($r = 0.697$, $p = 0.037$) and FAB ($r = 0.727$, $p = 0.041$) scores (Fig. 6C, D; left panel). The increase of brain-type Tf at post-operative month 12 was moderately correlated with MMSE ($r = 0.549$, $p = 0.01$).
(Fig. 6C, D; right panel). Scores for the modified Rankin Scale were not correlated with brain-type Tf levels before or after shunt surgery (data not shown). Furthermore, the increase of sAPP in both the pre- and post-operative periods was not correlated with MMSE or FAB. Concentration of L-PGDS was not measured due to limitation for CSF specimen available for the analysis. These results suggest that brain-type Tf levels at post-operative month 3 (and 12) predicts recovery from cognitive deficits in iNPH patients.23)

Spontaneous intracranial hypotension (SIH). SIH is commonly caused by leakage of the CSF. Patients with SIH often report orthostatic headache, dizziness, hearing disturbance, nausea and vomiting, and cervical pain. Cranial magnetic resonance imaging (MRI) of SIH patients revealed cranial hypotension-related manifestations such as diffuse pachymeningeal gadolinium enhancement, sagging of the brain, enlarged pituitary gland, descent of the brain stem, and subdural fluid collection. On radioisotope (RI) scintigraphy, findings include early accumulation of tracer in the bladder and kidneys, and a paucity of activity over the cerebral convexities. Myelography can show the location and extent of a CSF leak. While these abnormal findings are typical for SIH, 20–30% of SIH patients, however, do not show abnormalities in these clinical tests.32) We retrospectively studied CSF samples obtained by lumbar puncture of 62 consecutive patients suspected of intracranial hypotension. SIH was diagnosed based on the International Classification of Head Disorders, (3rd edition, beta version, http://beta.ichd-3.org/)

$p = 0.022$) but not with FAB ($r = 0.373$, $p = 0.154$) (Fig. 6C, D; right panel). Scores for the modified Rankin Scale were not correlated with brain-type Tf levels before or after shunt surgery (data not shown). Furthermore, the increase of sAPP in both the pre- and post-operative periods was not correlated with MMSE or FAB. Concentration of L-PGDS was not measured due to limitation for CSF specimen available for the analysis. These results suggest that brain-type Tf levels at post-operative month 3 (and 12) predicts recovery from cognitive deficits in iNPH patients.23)
and the diagnostic criteria reported by Schievink et al.\(^3\) In this way, 38 patients were classified as SIH and 24 patients as non-SIH. CSF samples were analyzed based on the groupings, along with the CSF samples of a neurological control group including patients with trigeminal neuralgia or unruptured cerebral artery aneurysm (n = 10).

Most CSF laboratory test results for the SIH and non-SIH groups were within reference intervals. Tests included osmolality, specific gravity, glucose concentration, chloride concentration, pH, and cell counts (leukocytes and erythrocytes). Only CSF protein concentration in the SIH was significantly higher than that of the non-SIH group (0.9 mg/mL versus 0.6 mg/mL, \(p < 0.001\)). In contrast, serum protein concentrations were not different between the two groups (\(p = 0.22\)). In the SIH group, the CSF protein concentration was not correlated with that of serum (\(r = 0.153, p = 0.367\)), suggesting that the increase in CSF protein level was independent of the serum protein levels.

To examine protein composition changes, CSF samples from the SIH and non-SIH patients were subjected to SDS-PAGE followed by silver staining (Fig. 7). Protein patterns were similar between the SIH and non-SIH groups, and composed principally of serum Tf (75 kDa), albumin (66 kDa), immunoglobulin heavy chain (Ig-H) (45–50 kDa area), and light chain (Ig-L) (25 kDa), L-PGDS, and TTR. While no apparent differences in protein composition were observed between the two groups, the signal intensity of each protein band, with the exception of the TTR bands, appeared to be increased in the SIH group. We therefore quantified these major proteins together with sAPP, a marker for CSF anomalies.

Albumin, IgG, total Tf, L-PGDS, TTR, sAPP, serum-type Tf and brain-type Tf were quantified by ELISA or Western blotting. Analysis of the data revealed that levels of these proteins, except for TTR, were significantly higher in the SIH than the non-SIH group (Fig. 7). TTR levels in the SIH and non-SIH groups were 24.8 µg/mL and 20.4 µg/mL, respectively, (\(p = 0.054\)). We performed statistical analyses on CSF proteins with a view to their potential use as differentiators (biomarkers) of SIH and non-SIH. Serum-derived proteins such as albumin, IgG, and serum-type transferrin showed differentiation sensitivities and specificities in the ranges of 76.3–78.9% and 70.8–79.2%, respectively, whereas the sensitivities and specificities of CNS-derived proteins such as L-PGDS, sAPP, and brain-type Tf ranged from 71.1–81.6% and 50.0–91.7%, respectively. Concentration analysis showed that changes in these markers were observed in the CSF of SIH patients, with sAPP levels being the highest among the measured proteins.

Fig. 7. Human CSF protein pattern for SIH and non-SIH patients. CSF proteins were analyzed by SDS-PAGE followed by silver staining. Arrowheads indicate the positions of serum transferrin (Tf), albumin (Alb), IgG heavy chain (Ig-H) and IgG light chain (Ig-L), respectively. Arrows indicate the position of lipocalin-type prostaglandin D synthase (L-PGDS) and transthyretin (TTR) purified from CSF.
Concentrations of serum albumin, IgG, and Tf were 40–70-fold higher than those in the CSF, suggesting that even slight contamination of blood disturbs the accurate estimation of the concentrations of these proteins. Based on these findings, we further analyzed CNS-derived proteins as preferred markers of SIH. Logistic regression analysis revealed odds ratios for L-PGDS, sAPP, and brain-type Tf of 9.15 (95%CI: 1.15–73.2, p = 0.036), 0.64 (95%CI: 0.13–3.1, p = 0.577), and 5.95 (95%CI: 1.01–35.1, p = 0.049), respectively. The combined use of L-PGDS and brain-type Tf differentiated SIH from non-SIH with a sensitivity of 94.7% and specificity of 72.6%.30

In SIH, the intracranial pressure decreases due to the leakage of CSF (threshold: less than 60 mm H2O). Moreover, the concentration of radioisotope (RI) injected into the CSF decreases rapidly and is excreted in the urine. The cutoff for RI residual activity at 24 hours after RI injection is set at 20%. With these clinical tests established to differentiate SIH from non-SIH, we therefore examined correlations between them and CNS-derived protein levels. Significant correlations were observed between the test outcomes and L-PGDS or brain-type Tf. A decrease in RI residual activity was inversely correlated with increases of L-PGDS (r = −0.56, p < 0.001) and brain-type Tf (r = −0.50, p < 0.001). Decreased intracranial pressure was also inversely correlated with increased of L-PGDS (r = −0.56, p < 0.001) and brain-type Tf (r = −0.46, p < 0.001). No other proteins were correlated with intracranial pressure or RI residual activity.30

**CSF marker changes in iNPH and SIH**

L-PGDS is mainly produced in the choroid plexus, leptomeninges, and oligodendrocytes, and then secreted into the CSF.36 It is then absorbed in the cranial arachnoid villi and flows into the dural sinuses.36 Mase et al. reported that L-PGDS levels were decreased in (idiopathic) normal pressure hydrocephalus.28 As described above, L-PGDS, brain-type Tf, and sAPP are all decreased in iNPH (Fig. 8B), where CSF production is also suspected to decrease. In contrast, these proteins increase in SIH (Fig. 8B), while CSF production is suspected to increase to compensate for CSF leakage. L-PGDS, brain-type Tf, and sAPP levels may thus change concomitantly with CSF production.

A possible explanation for the observed upregulation of brain-derived proteins in SIH patients is that the secretion of these proteins depends on CSF pressure or volume. As such, protein levels decrease in iNPH, in which the brain is compressed by excess CSF. As described above, brain-type Tf and sAPP were normalized or increased after shunt surgery, which bypasses excess CSF from the brain’s ventricles to the peritoneal cavity. This normalization suggests that CSF pressure or volume affect the secretion of the brain-derived proteins. Further research is required to clarify the exact molecular mechanisms underlying the increased levels of these proteins.

**Blood brain barrier (BBB) and protein permeability.** CSF is sequestered from blood by the BBB. As a result, the protein concentration of the CSF is about 100-fold lower than in the blood. The barrier, however, is leaky, even under normal physiological conditions. Indeed, about 80% of CSF proteins are derived from the blood, with this influx probably due to deficiencies in BBB structures. To this end, pericapillary lamellar processes of astrocytes are not observed in several paraventricular structures such as the posterior pituitary gland (neurohypophysis) and the pineal body.34 Under conditions of intracranial hypotension, the influx of different proteins depends on various factors such as their concentration in the blood, their molecular size, and interaction with other molecules. The increase of CSF protein levels in SIH is mainly attributable to albumin, which is biosynthesized exclusively in the liver and then secreted into the blood, suggesting that the increase in albumin is due to enhanced influx from the blood into the CSF. An enhanced inflow is also seen with IgG, which is mainly produced in the extra-neural lymphatic tissues. Other blood proteins such as serum Tf could flow into the CSF down their concentration gradient.30

**Conclusion**

We hypothesize that brain-type Tf secreted from the choroid plexus could be a biomarker of altered CSF production, and as such, useful for diagnosing iNPH. In addition, it may be also applicable to monitoring the normalization of CSF production after shunt surgery and for predicting cognitive recovery. This marker could also be useful for diagnosing SIH, where a marker with high sensitivity and specificity has not been available until now.

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Fig. 8. Box plots of protein concentrations in the CSF of SIH and non-SIH patients. Proteins examined were albumin, IgG heavy chain (Ig-H), serum-type Tf, L-PGDS, sAPP, and brain-type Tf (A). Horizontal lines within boxes show median values; boxes exclude upper and lower interquartile (IQ) ranges; whiskers indicate 1.5 times IQ: *p < 0.01, **p < 0.05, ***p < 0.001. CSF concentrations of L-PGDS, sAPP, brain-type Tf, and TTR in SIH (black bars) and iNPH (gray bars) are shown in (B): *p < 0.01, **p < 0.05, ***p < 0.001.
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Profile

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Profile

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