Journal of Trace Elements in Medicine and Biology 31 (2015) 183–187

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Toxicology

Iron and copper in progressive demyelination – New lessons from Skogholt's disease

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ARTICLE INFO

Article history:
Received 1 September 2014
Accepted 4 December 2014

Keywords:
Copper
Demyelination
Iron
Multiple sclerosis
Skogholt disease

ABSTRACT

The pathophysiological mechanisms of progressive demyelinating disorders including multiple sclerosis are incompletely understood. Increasing evidence indicates a role for trace metals in the progression of several neurodegenerative disorders. The study of Skogholt disease, a recently discovered demyelinating disease affecting both the central and peripheral nervous system, might shed some light on the mechanisms underlying demyelination. Cerebrospinal fluid iron and copper concentrations are about four times higher in Skogholt patients than in controls. The transit into cerebrospinal fluid of these elements from blood probably occurs in protein bound form. We hypothesize that exchangeable fractions of iron and copper are further transferred from cerebrospinal fluid into myelin, thereby contributing to the pathogenesis of demyelination. Free or weakly bound iron and copper ions may exert their toxic action on myelin by catalyzing production of oxygen radicals. Similarities to demyelinating processes in multiple sclerosis and other myelinopathies are discussed.

Introduction

The knowledge of causal mechanisms in demyelination is incomplete [1]. Several studies indicate that iron (Fe), copper (Cu) and other trace metals are involved in the pathogenesis of various neurodegenerative disorders [2–5] including the demyelinating disorder multiple sclerosis (MS) [6,7]. The potential neurotoxicity of Fe has been actualized by recent findings of Fe deposits in affected brain regions in Parkinson's disease [8,9] and in Friedreich's ataxia [10]. Copper neurotoxicity is illustrated by the neuropsychiatric symptoms characterizing Wilson's disease (WD) [11]. In WD, brain areas with Cu accumulation may be affected by demyelination [11]. Copper has been found in significantly elevated concentrations in cerebrospinal fluid (CSF) from patients with amyotrophic lateral sclerosis [12]. Recent studies have also indicated a pathogenetic role for Cu in Alzheimer's disease [13–16], evident by the copper-enrichment of the amyloid-beta-peptide plaques in the nervous tissue of Alzheimer patients [17,18], and in Parkinson's disease, evident by the specific Cu catalyzed oxidation of synuclein in Parkinson patients [19]. To what extent metal dyshomeostasis or elevated nerve metal concentrations can precipitate demyelination is however still unknown.

In a previous study [20] we found high levels of Fe and Cu in the CSF from patients with a disease which we named Skogholt disease. We have now made a clinical re-evaluation of that material, including new records of patient histories, resulting in exclusion of one patient, who had got his neurological symptoms from excessive drug abuse and who did not present demyelinated plaques when investigated with magnetic resonance imaging. The present updated discussion of the remaining nine patients is focusing on a possible pathogenetic role of Cu and Fe, in light of new insights from recent literature on Fe and Cu neurotoxicity.

Skogholt disease presents with demyelination of both the central and the peripheral nervous system. It was first described in 1998 by Dr. Jon Skogholt [21] in a region of Hedmark county in Norway. Clinically, it is characterized by a slowly progressing distal sensory loss, distal muscle weakness, unsteady gait and dysarthria.

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http://dx.doi.org/10.1016/j.jtemb.2014.12.002
0946-672X/

Table 1
Clinical characteristics of 9 patients with verified Skogholt disease.

Patient no.	Age (years)	Sex	Smoker	Activity	Education
1	40	m	Yes	Little	Elementary
2	32	m	Yes	No	High school
3	49	f	Yes	Little	Elementary
4	28	m	Yes	No	Elementary
5	44	f	No	No	High School
6	52	f	Yes	Yes	Elementary
7	55	f	No	Yes	Elementary
8	57	m	No	Little	Elementary
9	67	m	No	No	Elementary

Onset varies from the third to sixth decade in affected family members. As the disease does not conform to known hereditary demyelinating disorders it is considered a distinct disease entity [21]. Protein levels in CSF from these patients are substantially elevated [20], and a blood–brain barrier (BBB) dysfunction may be suspected.

The re-evaluation reported in the present paper confirms the previously reported large increase in intrathecal levels specifically of Fe and Cu in the Skogholt patients. CSF concentrations of the other elements analyzed were essentially similar to or marginally altered compared to those in the control group. We propose that Fe and Cu contribute to demyelination by oxidative mechanisms.

Materials and methods

Subjects

After a clinical reassessment of patients in our previous study [20], one patient was reclassified as he did not suffer from this disease. This resulted in 9 patients in the Skogholt group, 4 females and 5 males, mean age 47.1 years (Table 1). The control group consisted of 13 individuals without demyelinating neurological disorders [20], 8 females and 5 males, mean age 49.4 years. The project has been approved by the Regional Committee for Medical Research Ethics in Norway (Region East, ref. no. 556-04224).

CSF and blood sampling

Cerebrospinal fluid was collected by lumbar puncture with a standard Spinocan 0.9 mm × 88 mm syringe and transferred to metal free plastic tubes. Blood samples were collected from the cubital vein into heparinized vacutainer tubes specially designed for trace element analyses (Becton Dickinson).

Analytical methods have been described in detail previously [20] and are briefly outlined here. Metals were determined in CSF and blood plasma using high resolution inductively coupled plasma mass spectrometry (HR-ICP-MS). The accuracy of the method was checked by analyzing Seronorm Serum Level 1 and Level 2 reference material (Sero, Norway), showing values within 85–115% of the certified concentrations. The precision of the method, checked by repetitive analyses of the same sera, showed coefficients of variation lower than 10%.

Proteins in blood serum and CSF were quantified colorimetrically [20] and fractionated by electrophoresis. Concentrations of albumin were determined by an immunoturbidimetric assay (Tinaquant Albumin, Roche) analyzed on a Cobas c-501 instrument with CV 6%. IgG was quantified by the same method with a CV of 7%. The CSF IgG/total protein ratio and the CSF/serum protein ratio were calculated.

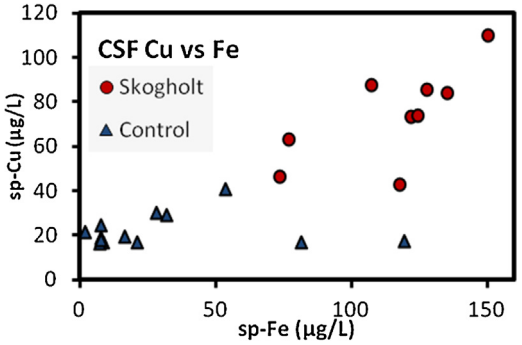

Fig. 1. Copper and iron concentrations (µg/L) in cerebrospinal fluid from individual patients with Skogholt disease (circles) and controls (triangles).

Statistics and graphic presentation

Graphs/plots were prepared in MS Excel 2007. For statistical evaluation, Pearson correlation coefficients and corresponding significance values were used. Differences between the concentrations and fractions for each group were tested using two-tailed two-sample *t*-tests. At $p < 0.05$ differences were considered significant.

Results

The present re-evaluation demonstrated an almost fourfold increase in both Fe (mean 115.2 µg/L) and Cu (mean 73.8 µg/L) concentrations in CSF from patients with Skogholt disease compared to Fe (mean 30.5 µg/L) and Cu (mean 21.7 µg/L) from controls (Table 2, Fig. 1). In CSF from Skogholt disease patients, Fe and Cu levels were correlated, but not very strongly (correlation coefficient, $r = 0.697$, cf. Fig. 1), which might indicate similar groups of carriers across BBB, e.g. peptides or proteins (see below).

CSF sulfur concentrations were substantially increased in Skogholt disease patients compared to controls, whereas a less pronounced increase was found for CSF zinc (Table 2). A modest increase in the levels of phosphorus and rubidium was observed, presumably not of independent pathogenetic significance. Only minor or negligible differences in CSF levels of the other elements, including manganese, lead and mercury, were found between Skogholt disease patients and controls (Table 2). The blood plasma levels for all measured elements including Fe and Cu in the Skogholt group did not differ from the control values (data not shown), suggesting a specific transfer of Fe and Cu into the CSF compartment. Concomitantly increased CSF Cu and S concentrations were found in the patient group compared to controls ($r = 0.937$) (Fig. 2), indicating that S-containing molecules, e.g. sulfur proteins, act as Cu transporters from blood to CSF in this group [20]. The same

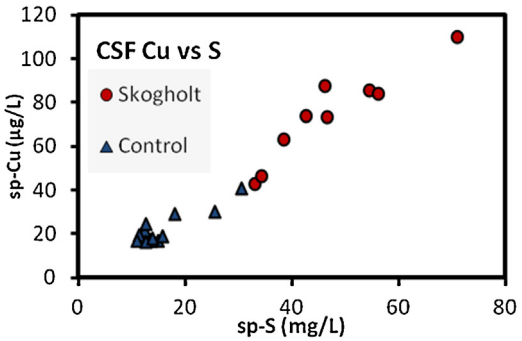

Fig. 2. Copper (µg/L) and sulfur (mg/L) concentrations in cerebrospinal fluid from individual patients with Skogholt disease (circles) and controls (triangles).

Table 2
Metal concentrations (mean values in μg/L) and standard errors of the mean (SEM) in cerebrospinal fluid from patients with Skogholt disease and controls, as measured by HR-ICP-MS. The ratio column displays concentrations in the Skogholt group divided by concentrations in the control group. The *p*-values (2-tailed *t*-tests) give significance for differences between the Skogholt group and the control group. Detection limits (d.l.) are defined as 3 × SD of blank samples.

CSF metal	Skogholt ($n = 9$)		Controls ($n = 13$)		Ratio	p
	Mean	SEM	Mean	SEM		
Iron	115.2	8.5	30.5	9.7	3.78	0.000
Copper	73.8	7.0	21.7	2.0	3.40	0.000
Sulfur	47,053	4009	15,927	1618	2.95	0.000
Zinc	44.2	4.2	24.6	2.5	1.80	0.001
Rubidium	90.5	5.9	65.4	3.8	1.38	0.003
Phosphorus	22,516	1053	19,076	596	1.18	0.014
Silicon	360	17.9	327	31.3	1.10	0.382
Calcium	60,144	6082	57,134	884	1.05	0.637
Magnesium	37,218	1677	36,589	548	1.02	0.729
Cesium	0.30	0.04	0.30	0.02	1.00	0.922
Strontium	15.71	1.4	15.87	1.4	0.99	0.940
Bismuth	0.025	0.014	0.028	0.0089	0.89	0.881
Yttrium	0.004	0.0008	0.005	0.0006	0.80	0.383
Thallium	0.05	0.0015	0.07	0.0050	0.71	0.000
Mercury	0.79	0.042	1.14	0.091	0.69	0.003
Uranium	0.002	0.0010	0.003	0.0008	0.66	0.558
Manganese	0.85	0.18	1.32	0.14	0.64	0.049
Molybdenum	0.26	0.022	0.42	0.097	0.62	0.135
Titanium	0.33	0.078	0.59	0.083	0.56	0.034
Chromium	0.11	0.034	0.20	0.050	0.55	0.127
Lead	0.28	0.10	0.59	0.068	0.47	0.026
Thorium	0.008	0.0014	0.021	0.0039	0.38	0.007
Cadmium	0.015	0.0052	0.040	0.0070	0.38	0.009
Vanadium	0.022	0.0099	0.059	0.039	0.37	0.373
Wolfram	<d.l.	–	0.024	–	–	–
Cerium	<d.l.	–	0.002	–	–	–
Gold	<d.l.	–	0.023	–	–	–
Cobalt	<d.l.	–	<d.l.	–	–	–

carrier(s) apparently did not to the same extent transport Fe across BBB since S correlated weaker with Fe ($r = 0.775$). Albumin, the main protein in the CSF, is a sulfur-rich protein. CSF albumin levels were greatly elevated in Skogholt disease patients, but CSF Cu ($r = 0.309$) and Fe ($r = 0.143$) did not correlate significantly with CSF albumin.

The albumin ratio between CSF and serum in Skogholt disease patients was increased more than threefold ($p < 0.00005$) compared to controls (Table 3). In general, the CSF/serum albumin ratio is a reliable indicator of BBB integrity [22]. Apparently, a BBB dysfunction is present in Skogholt disease. It is also noted that the CSF IgG-to-total protein ratio was virtually identical between the Skogholt disease group and the control group indicating that intrathecal IgG production does not characterize Skogholt disease as it does in MS. The CSF albumin/total protein ratio was lower in Skogholt disease patients than in controls. Apparently the non-albumin non-IgG protein fractions in CSF of Skogholt disease patients are higher ($p < 0.05$) than in the control group.

Discussion

The main finding in the present re-evaluation is an almost fourfold increase in Fe and Cu concentrations in CSF from patients with Skogholt disease compared to controls. A less pronounced increase in the levels of zinc may accentuate possible neurotoxic effects of Fe and Cu. The present discussion is focused on possible causes for, and consequences of the intrathecal Fe and Cu elevations.

By what mechanisms are iron and copper transferred from blood to CSF?

It is plausible that Skogholt disease is caused by an inherited defect that affects BBB integrity, leading to increased transfer of proteins, at least proteins of relatively low molecular weight, from blood to CSF [20]. An obvious carrier candidate for the transfer of Fe across the BBB into CSF is transferrin. With a molecular weight of only about 80 kDa, this protein is presumed to penetrate easily across a compromised BBB following the concentration gradient from blood to brain. Thus, transferrin is probably an important iron-binding component in the increased low molecular weight non-albumin non-IgG protein fractions observed in these patients (Table 3). In contrast, metals such as lead, mercury or zinc which are mainly trapped in red blood cells or incorporated in macromolecular proteins, do not penetrate to the same extent into the CSF, not even in Skogholt disease patients. With regard to a further penetration of Fe species into the cerebral white matter of these patients, it is relevant to note that oligodendrocytes and myelin are rich in transferrin receptors [23], rendering white matter susceptible to Fe overload. Furthermore, localized brain Fe accumulations, as observed in MS, may be implicated in the pathogenesis of demyelination [24–30].

The most important copper-transporting protein in blood is ceruloplasmin. With a molecular weight of above 150 kDa this protein does not easily penetrate an intact BBB. The strong correlation between Cu and S seen in Skogholt disease patients suggests that a sulfur-containing ligand or ligands bind Cu in the CSF in these patients. Apparently, this ligand is neither albumin nor IgG since the values in CSF of these proteins correlated poorly with the Cu values. Since ceruloplasmin is a sulfur-containing protein, it is tempting to suggest that significant amounts of this molecule or its fragments can cross a compromised BBB in Skogholt disease, explaining the Cu–S-correlation found in the CSF. Interestingly, the concept of Cu-containing ceruloplasmin fragments has been implicated in the pathogenesis of Alzheimer's disease [13]. This is consistent with the idea of low molecular weight non-albumin non-IgG Cu-complexes playing a metal-carrying role in Skogholt disease.

Neither Fe-carriers nor Cu-binding carriers in the CSF are expected to bind the metal ions irreversibly, but allow for some dissociation and redistribution. A deeper transfer of the Cu moiety into the cerebral white matter may occur, and lead to increased amounts of Cu ions in myelin in Skogholt disease, exceeding the

Table 3
CSF-to-blood serum ratios for albumin and IgG concentrations in CSF for the Skogholt group and the controls. The CSF albumin-to-total protein ratios and CSF IgG-to-total protein ratios are also given. The (remaining) non-albumin non-IgG-fractions in CSF from the Skogholt group and the control group are calculated.

		Skogholt ($n=9$)		Controls ($n=13$)		p-value
		Mean	SD	Mean	SD	
Albumin ratio	CSF (mg/L):serum (g/L)	16.59	4.88	5.09	2.47	0.000
IgG ratio	CSF (mg/L):serum (g/L)	12.39	7.16	3.01	1.82	0.004
CSF IgG/albumin ratio	CSF (mg/L):CSF (mg/L)	0.16	0.07	0.14	0.04	0.475
CSF albumin fraction	CSF (mg/L):CSF (mg/L)	0.56	0.04	0.62	0.05	0.012
CSF IgG fraction	CSF (mg/L):CSF (mg/L)	0.09	0.04	0.09	0.02	0.812
Non-albumin non-IgG fraction	CSF (mg/L):CSF (mg/L)	0.35	0.06	0.30	0.06	0.048

p-values given for Skogholt versus control group (two-tailed t-test).

detoxifying capacity of localized chaperones. Small molecules like amino acids [31] that form exchangeable metal complexes [32,33], can facilitate metal ion transfer across the barriers to vulnerable sites in myelin, and cellular uptake of free Cu ions may also occur [34].

A direct toxicity from these metal ions towards the barriers between blood and brain, facilitating ion transfer and increasing albumin, is another possibility that needs consideration [35]. Copper is classified as a selective choroid plexus toxicant while Fe belongs to the category of sequestered choroid plexus toxicants [36].

May raised iron and copper levels precipitate demyelination?

Demyelination is a characteristic finding in Skogholt disease patients. Free radicals formed in myelinated neurons have previously been implicated as causative agents in demyelinating processes [14,37–39]. It should be emphasized that not only free ions of Fe and Cu but also some chelates of Fe can catalyze the formation of reactive oxygen species (ROS), with myelinopathy as a possible consequence [40,41]. Such distortions make myelin more prone to attacks from macrophages [38]. It is of interest here that WD patients also have an approximately threefold increase in CSF Cu [42]. This leads in WD to a 3- to 4-fold increase in brain Cu levels associated with degenerative and demyelinating lesions [11]. Interestingly, WD patients without neurological symptoms do not show elevated CSF Cu [42], and upon Cu chelation in neurological WD the neurological symptoms and CSF Cu concentrations subside. Furthermore, autopsy studies in neurological WD have demonstrated multifocal demyelination and central pontine myelinolysis [43] emphasizing a role of myelin injury in this Cu toxicity disorder. Neuroradiological investigations have also demonstrated white matter lesions in neurological WD [44,45], consistent with demyelination.

Oxidative stress accompanying dysmetabolism of redox-active metals like Fe and Cu has been observed in several neurodegenerative disorders [3,4,8,10–12,14,46–49]. Also, deficiency of Cu, presumably accompanied by deficient function of the Cu scavenging enzyme SOD-1, may lead to myelinopathy [50]. In the cuprizone mouse model for MS, the copper-chelating agent cuprizone carries Cu into the CNS and causes widespread demyelination, strengthening the idea of demyelination being mediated by Cu dysmetabolism and ROS [51–58]. Simultaneous presence of poorly liganded Fe and Cu in the tissue will promote the conversion of nontoxic oxygen species to ROS through the Fenton reaction. The *simultaneous* presence of poorly liganded Fe and Cu in nervous tissue accelerates a catalytic generation of free radicals through the Fenton reaction [14,37]. Reactive oxygen species may by these mechanisms contribute to demyelination.

Does progressive demyelination include oxidative damage?

Demyelination in MS is presumably associated with autoimmune reactions. Progression of the disease seems to be influenced by oxidative stress, since the invasion of immune cells is accompanied by release of soluble factors including cytokines and ROS [59]. Several recent studies have reported peroxidative processes during the progression of MS [60–62], also in conjunction with metal exposure [63]. A presumption precipitating from the present and previous studies is that myelin is susceptible to ROS toxicity. If ROS mediated demyelination represents the white matter lesions in Skogholt disease [21], and if ROS toxicity is involved in the autoimmune reaction cascade in MS, metal induced ROS toxicity as a universal step in the mechanisms of progressive demyelination can be suggested. A corollary of this hypothesis is that therapeutic benefits from antioxidants and metal binding substances in the treatment of neurodegenerative diseases deserve further exploration [64,65].

Conflict of interest

The authors report no conflicts of interest.

Acknowledgements

Innlandet Hospital Trust is acknowledged for financial support. The HR-ICP-MS analysis was carried out by Syverin Lierhagen at the Norwegian University of Science and Technology (NTNU). The authors are indebted to the Department of Medical Biochemistry, Oslo University Hospital, Ullevål for carrying out protein determinations in cerebrospinal fluid. Krisitn Gellein, Eiliv Steinnes and Tore Syversen at the Norwegian University of Science and Technology (NTNU) and Gunnar B Thoresen at Innlandet Hospital are acknowledged for valuable advice.

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