Published in final edited form as:

Neuropathology. 2009 April ; 29(2): 187–195. doi:10.1111/j.1440-1789.2008.00943.x.

Iron localization in superficial siderosis of the central nervous system

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Abstract

Originally conceived as an uncommon disorder, with the advent of MRI, CNS superficial siderosis has been observed more frequently. We present histologic, histochemical, immunohistochemical, immunofluorescent and ultrastructural evaluation of a 56-year-old woman with superficial siderosis. Iron was concentrated in macrophages, superficial astrocytes and gray matter oligodendroglia deep within the cord. While spatially associated with dystrophic glial and neuronal spheroids, iron did not colocalize with mitochondria. Neurotoxic effects were observed despite selective iron localization only within a variety of non-neuronal cell types.

Keywords

brain; ferritin; iron; spinal cord; superficial siderosis

INTRODUCTION

Superficial siderosis of the CNS is an unusual disorder in which iron is deposited around involved structures in the brain, spinal cord, and some cranial nerves. Clinically, superficial siderosis manifests as a triad of hearing loss/tinnitus, cerebellar ataxia, and myelopathy, typically years after the initial bleed. A recent review of the literature revealed that 39% of patients present with all three components of the classical triad.¹ Other symptoms and signs include dementia, neurogenic bladder, anosmia, anisocoria, and anterior horn cell dysfunction.² A review of the causes of superficial siderosis showed that 19% of cases were attributed to trauma, including brachial plexus injury, 15% were due to tumor, 13% were secondary to previous surgery, 9% were due to arteriovenous malformations, and 35% were idiopathic in nature.¹ Examination of the CSF may show elevated protein, elevated numbers of red blood cells, and xanthochromia. Siderophages, erythrophagocytosis, and elevated ferritin and iron levels may be seen in the CSF as well.³ However, due to the episodic nature of subarachnoid hemorrhage in these patients, the CSF may appear normal as well.

Hamill has been credited with the first description of this entity in 1908, although this report lacked detailed histologic evaluation.⁴ Later reports, such as that by Lewey gave a more extensive histologic description of this entity.⁵ Previously considered a rare entity, with the advent and widespread use of MRI, superficial siderosis has been observed more frequently. As of mid-2006, approximately 270 cases of superficial siderosis of the CNS have been reported.¹ Given the distribution of iron and its effect on the magnetic properties of protons in effected tissues, spin echo T2-weighted and gradient echo T2-weighted MRI show a hypointense, black rim around involved structures.⁶ This change can be subtle and may be

overlooked. Of the two T2 sequence types, gradient echo is considered more sensitive.⁷ Imaging may also show cerebellar atrophy, especially in the superior vermis and the anterior cerebellar hemispheres, or atrophy of the spinal cord.⁸

In this report, we present a 56-year-old woman who was found to have superficial siderosis involving portions of the cerebrum, brainstem, cerebellum, and spinal cord. Our histologic, histochemical, immunohistochemical, immunofluorescent, and ultrastructural evaluation of the patient's CNS suggests an unexpected cellular distribution of the iron with implications on the pathogenesis of neurotoxicity in this disorder.

Clinical report

The patient was a 56-year-old white woman who initially presented in 1998 with a 6-month history of progressive visual loss and intermittent frontal headaches, fatigue, forgetfulness, and personality change. MRI revealed a 2-cm enhancing suprasellar mass that was biopsied and found to be a papillary craniopharyngioma. A bifrontal craniotomy was performed with subtotal resection of the tumor. Postoperatively, her course was complicated by symptoms of panhypopituitarism including diabetes insipidus, hypothyroidism, and hypogonadism. Two additional resections were performed for tumor recurrences at later dates (2001 and 2005) as well as two gamma knife surgeries. Prior to her final resection in December 2005, an MRI showed recurrent tumor and diffuse hemosiderosis, which was a new finding since her previous examination in March 2002 (Fig. 1). Three months after this resection, she experienced difficulty walking and mild confusion. These complaints were in addition to chronic complaints of blurred vision, headaches, and occasional tinnitus. At that time it was noted that she was unable to recall three objects on exam after 5 min, and walked with a wide-based gait. In March of 2006 she developed dizziness, new onset atrial fibrillation, peripheral edema and confusion, hypersensitivity to vibration, as well as episodes that resembled absence seizures.

Over the course of her disease, 11 CSF samples were obtained, all of which showed xanthochromia. CSF proteins ranged from 115 to 706 mg/dL, white blood cell count from 6 to 394/mm³, and red blood cell count from 2 to 5200/mm³. Imaging revealed no definite change in her tumor, but her mental and functional status continued to deteriorate. Mini Mental State Examination scores ranged from 26/30 to 20/30. During her final neurologic exam in October 2006, the patient's caregiver reported that the patient suffered from difficulties in concentrating and poor recent and day-to-day memory. She did not complain of vision, hearing or swallowing difficulties. On examination, she was noted to have a slow latency of response when questioned. Her language was normal. She had a mask-like face, a smooth gait when walking with a walker, and intact reflexes.

Approximately 8 years after her initial presentation, the patient experienced a grand-mal seizure followed by hemodynamic and neurologic deterioration. Brain death was declared 2 days later. An unrestricted autopsy was performed. The brain weighed 1400 g and was markedly soft and friable, with cerebellar tonsillar herniation and disruption of the brainstem. There was marked red-brown discoloration of the inferior portions of the temporal lobes, the anterior pons, the cerebellum, and the entire spinal cord. Residual craniopharyngioma was not present. The systemic autopsy was unremarkable.

MATERIALS AND METHODS

Complete neuropathological examination of brain and spinal cord was performed. Formalin fixed, paraffin embedded (FFPE) sections were evaluated by HE and Prussian Blue histochemical stains. Luxol Fast Blue stains were also performed on the spinal cord. Diaminobenzidine enhancement of the Prussian Blue reaction product was attempted, but did not show enhanced sensitivity.

Immunohistochemical stains for GFAP and neurofilament were performed using the biotin-streptavidin complex method. A hematoxylin counterstain was used. Additional information regarding all primary antibodies used in this study is provided in Table 1. To determine which cells contained iron, FFPE sections of spinal cord were stained with Prussian Blue and immunohistochemical stains using primary antibodies to GFAP, CD68, carbonic anhydrase, phosphorylated neurofilament, and MAP2. These sections were deparaffinized and rehydrated. Endogenous peroxidase activity was inactivated with 3% H₂O₂ for 20 min and antigen unmasking was performed using antigen retrieval citra solution (BioGenex, San Ramon, CA, US). All the secondary antibodies used were from Jackson ImmunoResearch Laboratories (West Grove, PA, US). To further evaluate iron-containing cells, FFPE sections of spinal cord were immunofluorescently stained with primary antibodies to ferritin and one of the following: GFAP, CD68, carbonic anhydrase, and MAP2. To further evaluate characteristic structures observed in the specimen, FFPE sections of spinal cord were also immunofluorescently stained with antibodies to ubiquitin alone and ubiquitin and either GFAP, ferritin, or neurofilament. The tyramide amplification system (PerkinElmer Life Sciences, Boston, MA, US) was used for ubiquitin/ferritin dual-labelled sections as described previously.⁹ All immunofluorescently stained sections were analyzed by laser confocal microscopy (LSM 510; Zeiss, Jena, Germany). Digital images were captured and analyzed with LSM 510 3.2 software (Zeiss).

For electron microscopic examination of the specimen, formalin fixed tissue was post-fixed in 2.5% glutaraldehyde for 10 min then cut into 1 mm cubes. These were fixed in glutaraldehyde for an additional 50 min. The tissue was then washed in PBS. The specimen was then post-fixed in 1% Osmium tetroxide containing 1% potassium ferricyanide for 1 h. After post-fixing, the tissue was dehydrated and embedded in epon resin. After sectioning, the specimen was stained with uranyl acetate and lead citrate and examined at 80 KV on a JEOL 1011 transmission electron microscope.

For gold-labelled immunoelectron microscopy, deparaffinization, antigen unmasking and blocking were done as described earlier. GFAP staining was performed using a rabbit polyclonal primary antibody followed by a donkey anti-rabbit biotin-conjugated secondary antibody (1:200, Jackson ImmunoResearch laboratories, West Grove, PA, US) followed by 10 nm gold-labeled streptavidin (GE healthcare, Little Chalfop, Buckinghamshire, UK).

RESULTS

The brain showed microscopic regions of diffuse acute to subacute hypoxic/ischemic changes. The leptomeninges were thickened and fibrotic. Granular hemosiderin pigment was observed in the leptomeninges, the underlying cortex, and in perivascular locations. Prussian blue histochemical stains showed iron deposition in the superficial insula, the entorhinal cortex, the midbrain, and pons. In some superficial areas the iron was associated with astrogliosis and spheroidal eosinophilic structures.

The spinal leptomeninges also appeared thickened and fibrotic. HE stains showed circumferential hemosiderin deposition in the leptomeninges and in the spinal white matter of all levels examined. Hemosiderin was observed in the spinal gray matter, most notably in the anterior horns, and in perivascular spaces (Fig. 2A,B). Astrogliosis and spheroids were also present in both gray and white matter (Fig. 2H). Definite neuronal loss was not appreciated by qualitative examination. Luxol Fast Blue stains showed circumferential, peripheral myelin loss (data not shown). Prussian blue stains showed diffuse iron deposition in the spinal white matter, with heavier accumulation in the subpial region and around blood vessels. In the spinal gray matter, the anterior horns showed the heaviest iron deposition (Fig. 2C–E). Iron was also observed in the cytoplasm of medium-sized cells with eccentric ovoid nuclei that morphologically appeared to be astrocytes, and in cells with coarse, intensely staining granules

and smaller, oblong nuclei consistent with macrophages. Over half of the spheroids stained lightly positive for iron. Immunohistochemical stains for GFAP highlighted reactive astrocytic processes and cell bodies. Gliosis was prominent in both the dorsal and ventral horns as well as the subpial white matter. Additionally, the majority of spheroids were highlighted by GFAP immunostains. Neurofilament immunostains also highlighted a few spheroids as well as axons within the specimen.

Prussian blue histochemical stained sections were immunostained with CD68, GFAP, carbonic anhydrase, MAP2, or neurofilament-specific antibodies. Cells exhibited colocalization of Prussian blue positivity with CD68, GFAP, and carbonic anhydrase, indicating the presence of iron accumulation in macrophages/microglia, astrocytes (Fig. 2F), and oligodendroglia. Iron did not colocalize with the neuronal markers MAP2 (Fig. 2G) or neurofilament. Neurofilament stains showed a relative thinning in the density of axons in the peripheral regions of the spinal cord. Many of the remaining axons in the periphery of the spinal cord appeared swollen and distorted. There was no loss of axonal density in spinal nerve roots by qualitative examination (data not shown).

Confocal fluorescent microscopy for GFAP/ubiquitin and neurofilament/ubiquitin revealed similar findings to the conventional immunohistochemical stained sections: the majority of spheroids showed colocalization of ubiquitin and GFAP. Ubiquitin-positive spheroids also colocalized with ferritin. Only occasional spheroids showed colocalization of neurofilament and ubiquitin (Fig. 2I–L).

To determine what cells contained excess iron, confocal microscopy was performed on sections of spinal cord stained with immunofluorescent stains for ferritin and CD68, GFAP, carbonic anhydrase, and MAP2. Ferritin colocalized with CD68-positive cells (Fig. 3A), and GFAP-positive cells confirmed the presence of iron in macrophages/microglia, and astrocytes. Some of the ferritin positivity colocalized with carbonic anhydrase-positive cells, particularly in the gray matter (Fig. 3B). Ferritin did not colocalize with the neuronal marker MAP2 (Fig. 3C).

Electron microscopy was performed on spinal gray matter and subpial white matter. It revealed electron-dense material, possibly representing iron, in cells with abundant amounts of intermediate filament (Fig. 4A). Gold-labelled immunoelectron microscopy showed the intermediate filaments to be GFAP positive, again confirming the astrocytic nature of these cells (Fig. 4B). Electron dense material was also seen in cells that morphologically resembled oligodendroglia, showing round nuclei and myelin connections to adjacent axons (Fig. 4C). While some cells showed structures that resembled mitochondria in various states of condensation with electron-dense material (Fig. 4D), double-label immunofluorescence for ferritin and mitochondria showed no colocalization (Fig. 3D).

DISCUSSION

After some initial speculation regarding superficial siderosis as a manifestation of hemochromatosis in the CNS, it is now generally accepted that this disorder is due to repeated, small, often asymptomatic subarachnoid hemorrhages. This has been experimentally reproduced by Koeppen and colleagues in rabbit experiments in which small amounts of autologous blood was injected into the subject's subarachnoid space.¹⁰ Over time, this hemorrhage results in increased heme in the CSF, which in turn, is broken down by heme oxygenase to form free iron and biliverdin. Free iron causes iron regulatory protein to disassociate from ferritin mRNA, resulting in ferritin production.¹¹ Ferritin serves as the primary intracellular iron storage protein, helping to protect the cell from the deleterious effects of excess free iron. With time, different subtypes of heme oxygenase are induced, and ferritin production shifts from the heavy subunit to the light subunit of ferritin. These changes in heme

oxygenase and ferritin production are thought to be key pathologic steps in the development of this disorder. Additionally, the process of iron release from heme and sequestration in ferritin and subsequently hemosiderin is thought to be first evident in perivascular cells, macrophages and microglia,¹² but iron has also been described in astrocytes¹³ and Bergman glia.¹⁴

Superficial siderosis can be focal in its involvement of CNS structures, or it can diffusely involve the entire neuraxis. The inferior temporal lobes, brainstem, cerebellum, periventricular structures, and spinal cord are preferentially involved, along with cranial nerve VIII. Microscopically, the leptomeninges typically appear fibrotic and show hemosiderin granules in macrophages and meningotheial cells.¹⁵ The cerebellum exhibits characteristic changes consisting of scarring and necrosis of the tips of the folia, with loss of Purkinje cells and granular neurons. The cerebellar sulcal depths are relatively spared. Bergman gliosis and microglial prominence have also been described.¹⁵ The changes observed in the cerebral cortex and spinal cord are typically less destructive in nature, consisting of gliosis, subpial hemosiderin deposition, and loss of superficial fibers. The central gray matter of the spinal cord may also show hemosiderin deposition. Granular ependymitis, subependymal glial proliferation, and hemosiderin deposition are seen in association with paraventricular structures.¹⁶

As others^{10,17} have, we used ferritin immunohistochemistry to define brain iron stores. Ferritin colocalized with CD68, and GFAP, representing macrophages/microglia and astrocytes. Ferritin also colocalized with oligodendroglial cells in the spinal gray matter, but not in spinal white matter. Oligodendroglia are thought to be iron “sinks” within the CNS, and in the normal adult brain, oligodendroglia have been shown to be the most common cell to stain for iron.¹⁸ Other investigators have shown oligodendroglia in the spinal white matter to stain more positively for iron than gray matter oligodendroglia in pathologic and non-pathologic states.¹⁹ Iron has been shown to accumulate in neurons in other iron-related disorders affecting the CNS.²⁰ Some authors have reported the same within neurons in siderosis,¹⁶ but this was not confirmed with colocalization of ferritin and neuronal markers MAP2 or neurofilament in the current study.

In this case, the distribution of iron in the spinal cord appears to follow two patterns. Superficial/subpial iron accumulation is commensurate with direct exposure to heme-laden CSF. However, this cannot explain the relatively intense iron deposition in the spinal gray matter. Furthermore, the colocalization of ferritin with oligodendroglia in the spinal gray matter, but not in the spinal white matter, suggests some factor other than simple diffusion from CSF into superficial structures may be at work. One possible explanation is similar to that put forward by Koeppen and Dentinger regarding Bergman glia in the cerebellum.¹⁴ A subset of cells located in the spinal gray matter may possess processes that extend to the pial surface, facilitating iron accumulation in this area.

The round-to-oval eosinophilic structures have been variously referred to as ovoid bodies,²¹ spheroids,^{17,22} cytooid bodies,²² grumose or foamy spheroid bodies,²³ or saccules and are seen in superficial siderosis and other disorders resulting in iron accumulation in the CNS, such as Hallervorden-Spatz syndrome, aceruloplasminemia, around older hemorrhages and in other neurodegenerative disorders. By their swollen and ubiquitinated nature, spheroids appear to represent dystrophic cellular processes and are felt by many to represent axonal swellings. However, some investigators describe similar structures as astrocytic in nature.^{23,24} In this study, we observed all of these structures to stain for ubiquitin. Additionally, many of these structures stained for GFAP, and a few showed positive staining for phosphorylated neurofilament. The spheroids appear to represent two types of structures: One is derived from swollen axons, and another is of astrocytic origin. In our case, the vast majority of these ubiquitin-positive dilated processes appeared astrocytic in nature.

On ultrastructural analysis, degenerating mitochondria were observed in various states of involvement by electron-dense material, possibly representing iron. Mitochondria are known to be targets of iron toxicity and oxidative damage. This is thought to be due in part to an already elevated level of free radical formation from normal cellular respiration. Excess free iron results in membrane peroxidation, damage to respiratory enzymes, and possibly damage to mitochondrial DNA.²⁵ Additionally, iron has been shown to accumulate in mitochondria in disorders such as sideroblastic anemia,²⁶ erythropoietic porphyria, hemochromatosis, Friedreich's ataxia, possibly thalassemic patients with iron overload, and others.²⁷ Despite these ultrastructural findings, ferritin did not colocalize with immunofluorescence for mitochondria. This may be due to antigenic loss in the degenerating mitochondria, or some other process preventing antibody binding.

In summary, this patient had longstanding superficial siderosis due to chronic CNS hemorrhage. The spinal cord demonstrated abundant sequestration of iron within macrophages/microglia, astrocytes, and oligodendroglia in the spinal gray matter. Electron microscopic analysis suggests the intracellular iron may accumulate, in part, in mitochondria, as is seen in other disorders. Also observed were iron-containing glial and neuronal spheroids of ubiquitinated proteins.

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Fig. 1.
Gradient echo T2-weighted MRI of the patient's spine shows circumferential signal hypointensity surrounding the spinal cord.

Fig. 2.

(A) HE staining. Leptomenigeal fibrosis and superficial hemosiderin deposition (arrowheads). (bar = 50 μ m) (B) HE staining. Hemosiderin deposition was observed in the spinal gray matter, such as this anterior horn that also shows numerous spheroids (arrowheads) and astrogliosis. (bar = 50 μ m) (C) Prussian blue staining. Diffuse iron deposition in both spinal gray and white matter. More intense positivity is observed in the subpial white matter and in the anterior horns. (bar = 2 mm) (D,E) Prussian Blue staining. A higher power view showing iron deposition in the leptomeninges and subpial layer (D) and in anterior horns (E). (bar = 50 μ m) (F) Astrocytes stain for both GFAP (brown) and Prussian Blue. (bar = 50 μ m) (G) Immunohistochemical stains for neuronal marker MAP2 (brown) do not colocalize with

Prussian blue (bar = 50 μ m) (H) HE staining. Granular spheroids in spinal cord white matter. (bar = 50 μ m) (I) Spheroids stain positively for ubiquitin (green). (bar = 20 μ m) (J) The majority of spheroids show colocalization of GFAP (red) and ubiquitin (green). (bar = 20 μ m) (K) A few spheroids show colocalization of neurofilament (red) and ubiquitin (green). (bar = 20 μ m) (L) Ferritin (green) colocalized with ubiquitin (red) in spheroids (bar = 20 μ m).

Fig. 3.

(A) Immunofluorescent staining of ferritin (green) and CD68 (red) showed colocalization indicating iron accumulation in macrophages/microglia. (B) Immunofluorescent staining of ferritin (green) and carbonic anhydrase (red) showed colocalization in gray matter. This was not seen in white matter oligodendroglia. (C) Immunofluorescent double-staining for ferritin (green) and MAP2 (red) did not colocalize with the neurons. (D) Immunofluorescent of ferritin (green) and mitochondria (red) did not colocalize (bar = 40 μ m).

Fig. 4.

(A) Transmission electron microscopy. Electron-dense material is observed in cells with abundant amounts of intermediate filaments consistent with GFAP in astrocytes (bar = 500 nm). (B) Gold immunoelectron microscopy for GFAP shows gold particles bound to intermediate filaments in a cell containing electron-dense material (bar = 500 nm). (C) Electron-dense material was also observed in oligodendroglial cells (bar = 500 nm). (D) Some cells contained structures that resembled mitochondria showing various degrees of involvement by electron-dense material (bar = 500 nm).

Table 1
Antibodies used in this study

Antigen	Catalog number	Antibody type	Dilution	Source
Immunoperoxidase				
GFAP	Z0334	Rabbit polyclonal	1:500	DAKO (Carpinteria, CA)
CD68	M0814	Mouse monoclonal	1:100	DAKO (Carpinteria, CA)
Carbonic anhydrase	AF2184	Sheep IgG	1:100	R&D Systems (Minneapolis, MN)
Neurofilament	M0762	Mouse monoclonal	1:100	DAKO (Carpinteria, CA)
MAP2	SMI 52	Mouse monoclonal	1:500	Sternberger Monoclonals (Lutherville, MD)
Immunofluorescence				
GFAP	Z0334	Rabbit polyclonal	1:500	DAKO (Carpinteria, CA)
Ferritin	PA1-23700	Rabbit polyclonal	1:50	ABR (Golden, CO)
CD68	M0814	Mouse monoclonal	1:100	DAKO (Carpinteria, CA)
MAP2	SMI 52	Mouse monoclonal	1:500	Sternberger Monoclonals (Lutherville, MD)
Mitochondria	MU213-UC	Mouse monoclonal	1:50	BioGenex (San Ramon, CA)
Neurofilament	M0762	Mouse monoclonal	1:100	DAKO (Carpinteria, CA)
Carbonic anhydrase	AF2184	Sheep IgG	1:100	R&D Systems (Minneapolis, MN)
Ubiquitin	Z0458	Rabbit polyclonal	1:200	DAKO (Carpinteria, CA)