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Original Article

Hereditary diffuse leukoencephalopathy with spheroids: ultrastructural and immunoelectron microscopic studies

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Abstract: Hereditary diffuse leukoencephalopathy with spheroids (HDLS) is a rare autosomal dominant disorder characterized by cerebral white matter degeneration with myelin loss and axonal swellings (spheroids) leading to progressive cognitive and motor dysfunction. Histopathology of HDLS has been well characterized, but ultrastructural details are lacking. Here we report ultrastructural and immunoelectron microscopic characterization of spheroids and capillary basal lamina in white matter of HDLS brains. Spheroids had thin or discontinuous or no myelin sheaths. They contained various combinations of aggregated neurofilaments (NF), cytoplasmic organelles, dense bodies, and laminated figures. Aggregated filaments labeled with antibodies to phosphorylated NF (pNF), non-pNF and amyloid precursor protein. The gliotic white matter had many reactive astrocytes, and lipid-laden macrophages with membranous and fingerprint-like bodies. The basal laminas (BL) of many capillaries were dilated, and the enlarged space was heavily deposited with banded collagen type I and III. Some BL had focal thickenings and duplications. Fibronectin, not collagen IV, was found associated with banded collagen. The various types of axonal spheroids and changes in capillary basal lamina have not been emphasized previously. It remains to be determined if they are a reactive process or a primary mechanism of white matter degeneration in HDLS.

Keywords: Hereditary diffuse leukoencephalopathy, spheroids, capillary basal lamina, ultrastructure, immunoelectron microscopy

Introduction

Hereditary diffuse leukoencephalopathy with spheroids (HDLS) is a rare autosomal dominant disorder characterized by cerebral white matter degeneration with loss of myelinated axons and axonal spheroids (swellings) leading to progressive cognitive and motor dysfunction. In 1984, Axelsson et al [1] first described this disorder in a large Swedish kindred with variable neurological presentation and with disease onset occurring in the 30's. Psychiatric symptoms were usually the first and predominant neurological manifestation of HDLS. The other frequently presenting symptoms included gait instability, incoordination and seizures. Neuropathological studies of 4 affected individuals in this kindred showed a widespread leukoencephalopathy characterized by loss of myelin sheaths and axons, and neuroaxonal spheroids in the affected white matter. Since then a handful of studies of familial and sporadic cases have been reported [2-13]. Despite variability of clinical symptoms, neuropathology of white matter degeneration described by light microscopy is very similar, but ultrastructural data have been sporadic [1, 2, 6, 9, 11-13]. All have shown spheroids, but only Axelsson et al. [1] mentioned changes in capillaries in the white matter.

In a previous report of a family with HDLS, immunohistochemistry (IHC) of three affected members showed that axonal spheroids were immunostained for phosphorylated neurofilament, amyloid precursor protein and variably for ubiquitin, but not for tau, α-synuclein or αB-crystallin [11]. Here we describe ultrastructural pathology and immunoelectron microscopic characterization of spheroids and capillary basal lamina in the white matter of HDLS that have not been reported in any of the previous studies.

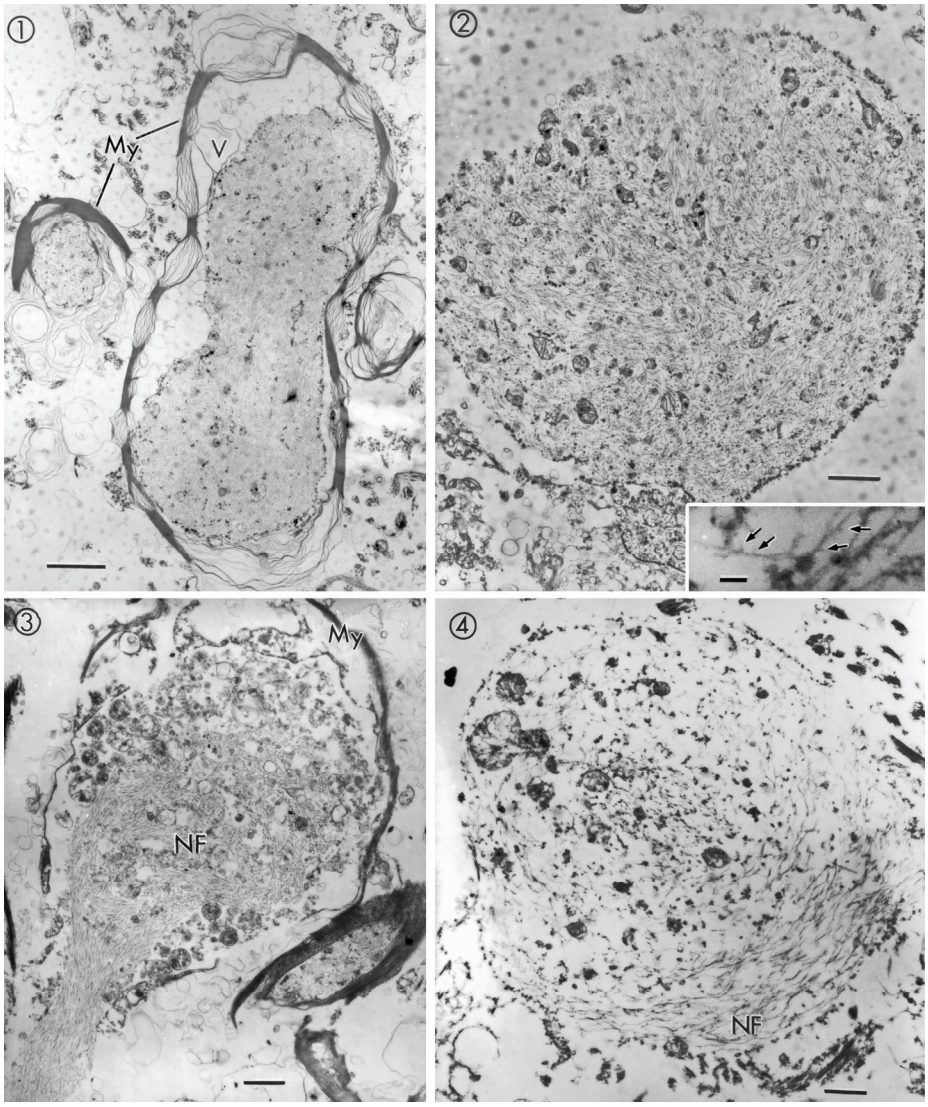


Figure 1. Two axons with similar thickness of myelin sheath (My). The swollen axon is packed with chaotic neurofilaments with trapped organelles. V, vacuolations of myelin sheath. Bar, 3 μm.

Figure 2. A spheroid with almost no myelin sheath is filled with numerous chaotic filaments trapping scattered mitochondria, small vesicles and membranous bodies. Bar, 2 μm. Inset, the filaments have diameters of 8-10 nm and side arms (arrows), characteristics of neurofilaments. Bar, 60 nm.

Figure 3. A spheroid with thin, discontinuous myelin sheath (My), packed neurofilaments (NF) and peripherally located organelles. Note the thickness of myelin sheath around a non-swollen axon. Bar, 1 μm.

Figure 4. An unmyelinated spheroid has peripheral bundles of neurofilaments (NF) surrounding organelles. Bar, 1 μm.

Materials and methods

Gray matter, underlying white matter and periventricular white matter of the superior frontal gyrus removed from formalin-fixed brains of six patients, aged 46, 49, 51, 55, 62 and 71 years, were processed for routine electron microscopy (EM) and post embedding immunogold EM (IEM) as previously described [14].

We used antibodies to the following proteins: amyloid precursor protein (APP), clone 22C11 (Chemicon, Temecula, CA); phosphorylated neurofilament M and H (pNF) (SMI 31), nonphosphorylated neurofilament H (non-pNF) (SMI 32) (Sternberger Monoclonals, Lutherville, MD); αB-crystallin (αB-c) (Dr. Jack Liang, Harvard University) [15]; glial fibrillary acid protein (GFAP) (BioGenex, San Ramon, CA); collagen types I, II, III and IV (Rockland, Gilbertsville, PA); fibronectin (Sigma, St. Louis, MO). 10 nm gold particles conjugated secondary antibodies were purchased from Amersham (GE Healthcare, Piscataway, NJ).

Results

Spheroids and glial cells

Spheroids had different thickness of the myelin sheath with severely swollen axons showing very thin or no myelin sheathes (**Figure 1-4**). Axoplasm in spheroids showed various combinations of organelles. Some were filled mainly

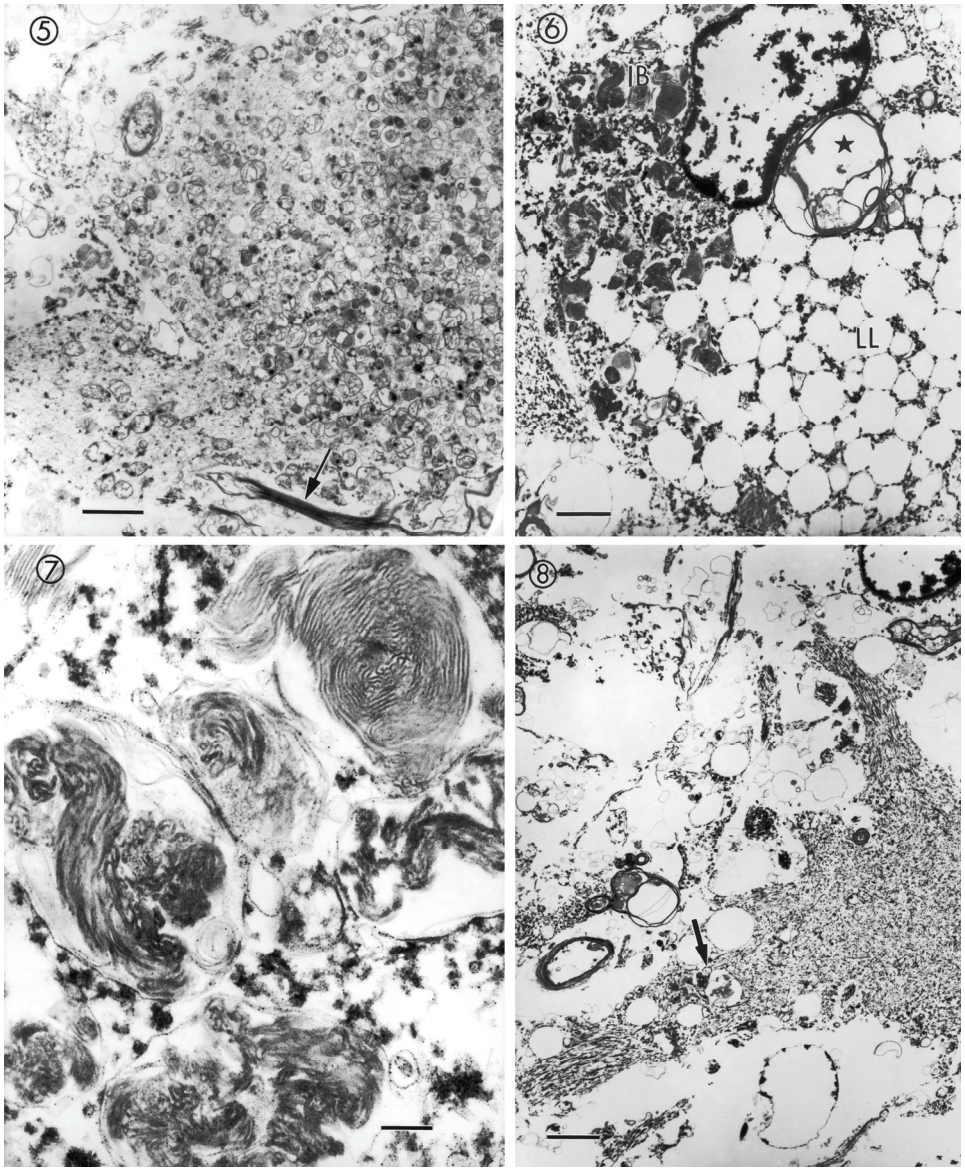


Figure 5. A spheroid with a fragment of myelin sheath (arrow) is filled with numerous vesicles, dense bodies, mitochondria and scattered clusters of neurofilaments. Bar, 2 μm.

Figure 6. A lipid-laden (LL) macrophage contains myelin debris (*) and many inclusion bodies (IB) that are enlarged in Figure 7. Bar, 2 μm.

Figure 7. Inclusion bodies in Figure 6 are membrane-bound lamellated or fingerprint-like structures. Bar, 0.3 μm.

Figure 8. A reactive astrocyte is expanded with numerous glial fibrils. Arrow points to lipofuscin granules. Bar, 3 μm.

with aggregated neurofilaments with trapped organelles, such as mitochondria and vesicles (**Figure 1, 2**). Others had the aggregated filaments either pushing the axoplasmic organelles to the periphery (**Figure 3**), or confining them centrally (**Figure 4**). Another spheroid type contained mainly dense bodies and vesicles, but very little filaments (**Figure 5**). These various spheroids were commonly found in severely degenerated white matter, and decreased gradually toward the U-fibers where axons retained normal myelin sheaths.

In severely degenerated areas there were many lipid-laden macrophages (**Figure 6**) with aggregates of membranous, lamellated or fingerprint-like bodies (**Figure 7**). Also common in these areas were many large reactive astrocytes filled with glial fibrils (**Figure 8**). Some reactive astrocytes and oligodendrocytes also contained lipofuscin granules.

IEM showed that filaments in spheroids were highly reactive to pNF antibody SMI 31 (**Figure 9**). A subset of spheroids had filaments moderately to weakly reactive to non-pNF antibody SMI 32 (**Figure 10**), which is normally found in cell body and dendrites, but not axons. APP immunoreactivity was also found associated with filaments in spheroids (**Figure 11**). αB-

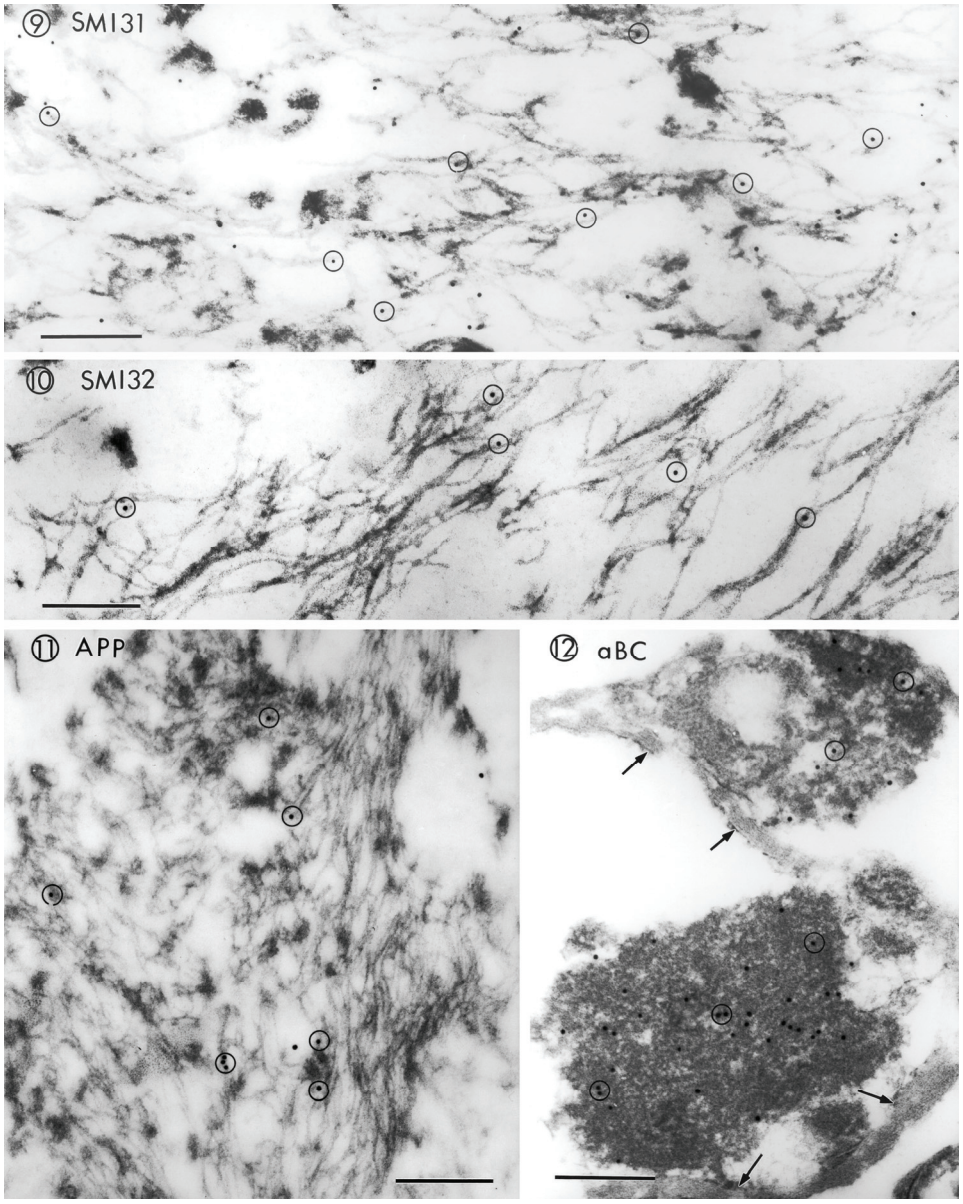
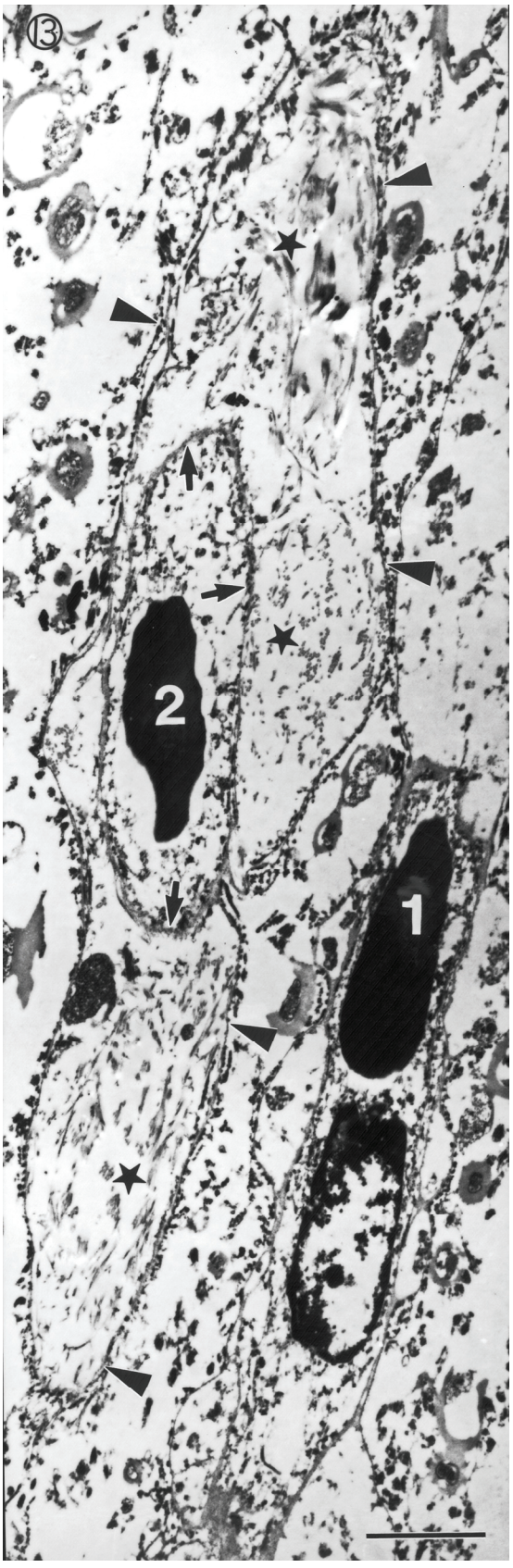


Figure 9-12. Immuo-EM of spheroids. Filaments in spheroids are strongly labeled with antibody SMI31 to pNF, only some gold particles are circled (Figure 9); weakly with SMI32 to non-pNF (Figure 10). Antibody to APP is found over filaments and associated dense material (Figure 11). αB-crystallin antibody (αBC) labels dense material in slightly swollen axons (Figure 12). Arrows point to myelin sheaths. Bars, 0.3 μm.

crystallin, which is generally absent from axons, was found in aggregates of dense, amorphous material in slightly swollen axons (**Figure 12**). APP, αB-crystallin and GFAP were also localized to glial fibrils in reactive astrocytes (data not shown).

In contrast to normal capillaries, many capillaries had markedly dilated basal lamina with the expanded space between the basal lamina of endothelial cells, pericytes and astrocytes filled by considerable deposits of banded collagen fibrils (**Figure 13**). Most fibrils had a banding periodicity of about 65 nm, and a diameter of less than 100 nm; however, some fibrils were more than 100 nm and had a ragged, moth-eaten appearance (**Figure 14**). These banded fibrils were positive for antibodies to type I and type III collagen (**Figure 15**), but negative for type II collagen. Type IV collagen was localized mainly to the amorphous, electron-dense basal lamina (**Figure 16**). In contrast, fibronectin was found not only in the amorphous basal lamina, but also associated with banded collagen deposited in the widened space between the basal lamina (**Figure 17**).



Discussion

Here we describe two major ultrastructural features of the white matter in HDLS: (1) axonal spheroids and (2) the changes in capillary basal lamina. The pathology was further characterized by IEM.

Spheroids contained various combinations of cytoplasmic components. Thus, some spheroids were filled with packed neurofilaments and a few trapped cellular organelles, such as mitochondria, ribosomes and vesicles, while others were packed with membrane-bound dense bodies and a few filaments scattered among them. Aggregation of filaments either pushed organelles peripherally or confined them centrally. Whether the various types of spheroids represent different stages of axonal degeneration remains unknown. It is also not clear whether the accumulation of neurofilaments is the cause or result of axonal swelling.

Our early study of HDLS showed immunohistochemical staining of abundant spheroids with pNF [11]. In this study, a subset of spheroids was immunoreactive to antibodies against non-pNF and αB-crystallin. Immunostaining of non-pNF to demyelinating axons and axon-terminal spheroids is thought to be indicative of axonal transaction in multiple sclerosis [16]. αB-crystallin is a member of heat shock proteins with diverse functions in the nervous system [17]. Its roles, if any, in HDLS remains to be determined.

Myelin sheaths of spheroids also showed various thicknesses. The larger (more swollen) spheroids appear to have thinner sheaths. This correlation could be confirmed by quantitative studies. Loss/thinning of myelin sheaths would result in decompaction of the sheath and subsequent swelling of axons and accumulation of axonal contents. EM also revealed vacuoles between axoplasm and myelin sheath, giving the appearance of peripheral vacuolations of

Figure 13. Two capillaries with different basal lamina morphology. Capillary 1 has normal, thin basal lamina (BL). In contrast, capillary 2 has dilated space (*) between BL of endothelial cells (arrows) and perivascular astrocytes (arrowheads). The space contains heavy deposits of collagen fibrils. Bar, 9 μm.

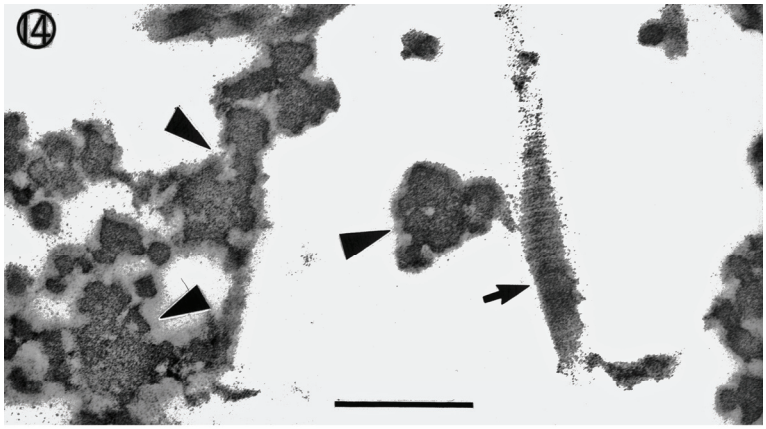


Figure 14. A regular-profile banded collagen fibril (arrow) and fibrils with larger diameters and irregular contour as well as a moth-eaten appearance (arrowheads). Bar, 0.3 μm.

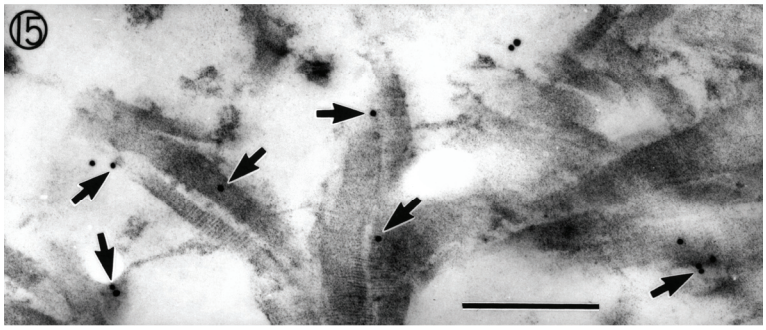


Figure 15. Immunogold labeling (arrows) of banded collagen fibrils with antibody to collagen type III. Bar, 0.3 μm.

spheroids by light microscopy. Since myelin sheaths are produced by oligodendrocytes, myelin pathology could be caused by oligodendrocyte degeneration. Transgenic mice overexpressing tumor necrosis factor (TNF)-α by astrocytes selectively induced oligodendrocyte apoptosis and myelin vacuolation in the absence of immune cell infiltration. The primary demyelination then evolves to have many phagocytic macrophages, axonal damage and markedly oligodendrocyte loss [18]. In vanishing white matter disease (VWM), a disorder clinically and pathologically similar to HDLS, white matter pathology has been shown to be due, at least in part, to oligodendrocyte dysfunction and loss [19, 20]. VWM has been linked to mutations in genes encoding the subunits of eukaryotic initiation factor 2B [21, 22], which were shown to be unaffected in HDLS in our previous study [11]. Additional quantitative and qualitative studies of oligodendrocytes are needed in HDLS.

It has been postulated that in Binswanger disease hypoperfusion of the deep white matter due to stenosis of the long penetrating arterioles causes demyelination, axonal loss and gliosis of the subcortical white matter or leukoencephalopathy [23]. In the first report of HDLS, Axelsson et al. [1] noted white matter capillary pathology in the form of duplicated basal lamina (BL). Their low magnification micrograph looked similar to ours. Here, we provide higher resolution images of common findings in capillary BL of HDLS. Normally, the vascular BL is a thin, amorphous structure that is formed by fusion of two basal laminae, each from the vascular endothelial cell/pericyte and the perivascular astrocyte [24]. In HDLS many capillaries in affected white matter showed the two basal laminae were widely separated and the widened space was deposited with large amounts of banded collagen and other material. Similar capillary ultrastructure has been reported in white matter of biopsy tissues from human leukodystrophies, including adrenoleukodystrophy, Canavan's disease and Alexander's diseases [25]. Therefore, these changes cannot be due to post mortem changes in autopsy tissues. Similar collagenosis in the human cerebral periventricular white mater (WM) has been shown to increase with age, notably after 70 years of age [26]. The ages of our cases were mostly younger than that (i.e. 46, 49, 51, 55, 62 and 71 years). Further, these changes were observed in white matter beyond the periven-

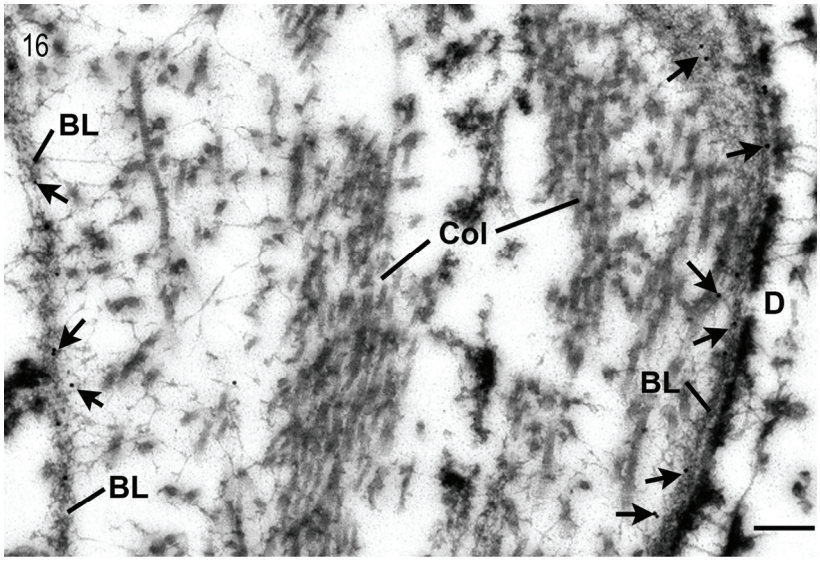


Figure 16. Immunogold labeling (arrows) of collagen type IV over basal lamina (BL), but not over banded collagens (Col) in the expanded space between BL. D, hemidesmosomes of astrocytic endfoot adjacent to BL. Bar, 0.2 μm.

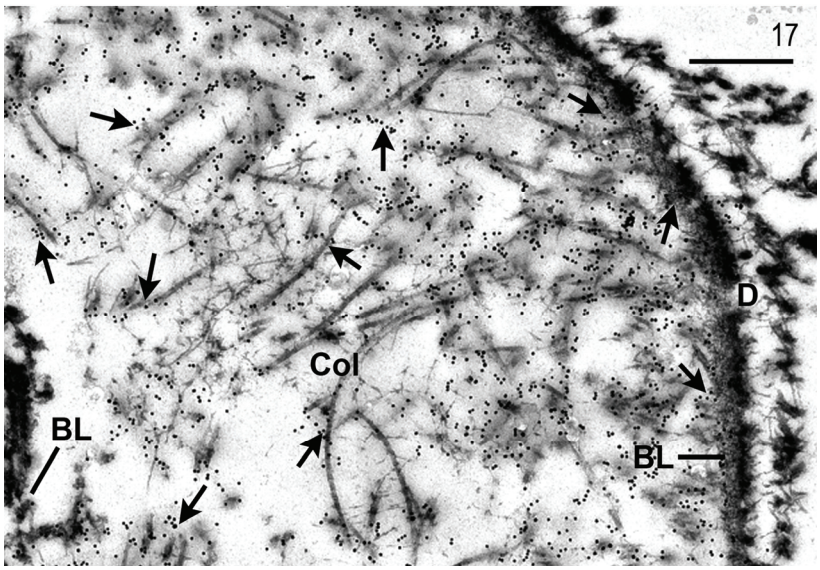


Figure 17. Heavy immunogold labeling (arrows point to some) of fibronectin over basal lamina (BL) and banded collagens (Col) in the expanded space between BL. Note hemidesmosomes (D) of astrocytic endfoot adjacent to BL. Bar, 0.5 μm.

tricular area in our cases, and in spinal cords of amyotrophic lateral sclerosis (ALS) cases with a mean age of 60.7± 7.8 years [27 and references therein], as well as a 15-year-old ALS patient [our unpublished data], suggesting that factor(s) other than old age may be involved. It is noteworthy that Kondo and Suzuki [25] also reported increased pinocytotic vesicles in the capillary endothelial cells and postulated that this and the widened capillary wall may be closely related to the disruption of blood-brain barrier in leukodystrophies. The banded collagen in HDLS has normal diameter (20 to 100 nm) and periodicity (65 nm). Occasionally, some fibers had diameters over 100 nm; others aggregated and showed ragged, moth-eaten appearance. Such appearance is thought to be due to dissociated or poorly packed collagen fibrils [28].

Previous studies did not identify the basal lamina components. Our IEM finding is the first to identify capillary banded collagen as being derived from type I and III collagen, which also had association with fibronectin, but not collagen type IV. A recent review lists collagen IV, laminin, nidogen and perlecan as four major components of basal lamina in general [29]. Fibronectin is not considered a component *per* se, due to its variable detection [29]. During

cerebral angiogenesis in the rat, fibronectin was down-regulated in capillaries [30]. It was strongly increased in adult mice subjected to hypobaric hypoxia [31], indicating angiogenic role for fibronectin. In Binswanger's disease, there is a marked deposition of several collagen types, including type I, in intracerebral arterioles and capillaries [32]. Accumulation of collagen could be due to increase synthesis or decreased degradation. Metalloproteinases (MMP) are enzymes involved in degradation of extracellular matrix, and MMP-2 and -9 degrade collagen types I and III [33]. Interestingly, MMP-2 activity was decreased, while MMP-9 activity was significantly increased in spinal cord of ALS patients [34], where we and others have seen deposits of collagen in capillary basal lamina. The significance of our findings with respect to cerebral capillary function in HDLS, and perhaps other neurodegenerative disorders, remains to be investigated.

On a technical note, there are some reports that immunolabeling of fibrillar collagen requires collagenase digestion to loosen up the interactions between collagens and other extracellular components. We were able to detect capillary associated collagen in paraffin embedded sections simply by heating in pH 7 citrate buffer (data not shown), while no pretreatments were needed for IEM, indicating that these collagens are probably not tightly bound with basal lamina components. This notion agrees with the loose and random distribution of the collagens fibrils in the widened basal lamina.

Ultrastructure of lipid-laden macrophages has not been reported in previous cases of HDLS. The ceroid-like ultrastructural appearance, with lamellated or fingerprint-like bodies, was similar to that reported in neuronal ceroid-lipofuscinoses [35] and orthochromatic leukodystrophy [36, 37] and may be nonspecific [38]. On the other hand, they appear to be a consistent finding in HDLS.

In summary, we describe ultrastructural pathology in white matter of HDLS. Spheroids of myelinated or demyelinated swollen axons contained packed pNF and variable non-pNF, mitochondria and cellular debris. Capillaries have split basal laminae with considerable deposits of banded collagen type I and III that were associated with fibronectin, but not type IV collagen. It remains to be determined if these changes are a primary cause or a reactive response of white matter degeneration in HDLS.

Acknowledgements

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