Studies on the retina and the pigment epithelium in hereditary canine ceroid lipofuscinosis

III. Morphologic abnormalities in retinal neurons and retinal pigmented epithelial cells

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Studies of the retina in 6- and 22-month-old English setters with progressive blindness, ataxia, and muscle weakness demonstrated a marked accumulation of abnormal cytosomes within neurons and retinal pigmented epithelial cells. Ganglion cells contained abundant cytosomes with evenly spaced stacks of membranes; bipolar and amacrine cell cytosomes consisted of dense, amorphous material with closely spaced configurations of light and dark lines; cytosomes within photoreceptor cells contained faintly staining curved profiles. All three cytosomes resembled those previously reported in brain neurons of CCL dogs. In retinal pigmented epithelial cells there were prominent accumulations of lamellar fragments, either free in the cytoplasm or incorporated into melanin granules. These retinal abnormalities are likely to be related to deficiencies of peroxidase and defects of lipid peroxidation. The pathologic and biochemical changes seen in these dogs are similar in many respects to those reported in human patients with Batten disease. As such, these dogs provide a convenient model for the study of disease mechanisms and for therapeutic approaches to blindness in Batten disease.

Key words: retinal neurons, retinal pigmented epithelial cells, cytosomes, fingerprints, curvilinear bodies, peroxidase deficiency, lipid peroxidation defect, ceroid, Batten disease

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Human ceroid lipofuscinosis, or Batten disease, is marked by blindness, dementia, limb ataxia, and premature death. On the basis of the few cases reported where the eye was studied microscopically, blindness results from a loss of photoreceptor elements, including rods, cones, cell bodies of the outer nuclear layer. Neurons in remaining retinal layers contain lipopigments characteristic of the disease, and the retinal pigmented epithelium may be atrophic or totally lost. The ERG is usually absent, and fluorescein angiography is grossly abnormal, especially in the macular region. The cause of abnormal lipopigment accumulation and photoreceptor cell death is not understood. Because of its rarity, there are few detailed...
studies of retinal abnormalities in human Batten disease. Consequently, investigators have sought an animal model for studies on the mechanism of this disease and for use in testing possible modes of therapy. Recently such a model, canine ceroid lipofuscinosis (CCL), has been suggested. These reports describe a breed of English setter developing clinical abnormalities and showing light and electron microscopic changes in nervous tissue similar to those reported in human Batten disease. An abnormal deficiency of leukocyte peroxidase has been reported in both the human disease and the canine disorder. To date, no detailed morphologic description of the retina in the canine model has been published. It is the purpose of this communication to report light and electron microscopic changes in retinal neurons and retinal pigmented epithelial cells in these animals.

Methods and materials

The dogs studied were from the colony of English setters in which the disorder was first observed. As in previous experiments, affected and normal dogs were identified by brain biopsy at age 3 to 4 months; they were flown from Oslo, Norway, to Colorado and kept in the Animal Care facility at the University of Colorado Medical Center.

In this study five "young" dogs (age 6 to 8 months) and five "adult" dogs (age 22 to 24 months), all from the English setter colony, were examined. There were four affected (hereafter termed CCL dogs) and one normal littermate in the "young" group and three affected (CCL dogs) and two normal dogs in the "adult" group. Over a 4-week period prior to sacrifice, animals were ex-
glomerular abnormalities

Retina in CCL: morphologic abnormalities

Fig. 2. Higher-power view of cytosomes within a ganglion cell. Curved membranes stacked at regular intervals appear in configurations resembling fingerprints (several marked with arrows). A unit membrane invests each cytosome. Insert: Detail of fingerprint which is composed of trilamellar membranes stacked at 4 to 6 nm intervals. The membranes are not as distinct as the unit membrane investing the cytosome (small arrow). Bar indicates 0.1 μm.

amined clinically on multiple occasions, underwent electroretinography one or more times, and had blood drawn for white blood cell peroxidase determinations.

Animals were sacrificed by electric shock and the eyes quickly removed. The cornea and iris were cut away and the vitreous expressed by gentle pressure. For electron microscopy a 5 mm corneal trephine was used to remove a single, circular block of intact full-thickness retina-choroid-sclera. Specimens were processed immediately as described below.

Electron microscopy. The specimens obtained by trephine cut were placed immediately in phosphate-buffered 4% glutaraldehyde and fixed for 24 hr. Under a dissecting microscope small specimens were cut, rinsed in phosphate buffer, osmicated, dehydrated, and embedded in Araldite. Semithin sections (1 μm) were cut and stained with toluidine blue for examination by light microscopy. After selection of appropriate areas, thin sections (600 to 700 Å) were cut, mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined in a Philips 300 electron microscope.

Results

Clinical observations. The 6- and 8-month-old young CCL animals were identical in all respects to their control littermates. Only the results of a previous brain biopsy allowed precise identification of the affected animals. In contrast, the three "adult" CCL animals were quite abnormal. Two had a peculiar, unsteady gait marked by frequent falls. Attempts to feed were characterized by lunging movements of the muzzle which repeatedly overshot or otherwise missed the target. Reduced visual acuity was obvious by the dogs' lack of response to visual threat. The third

Fig. 3. Inner nuclear layer, 22-month-old CCL dog. Multiple, darkly staining cytosomes (arrows) are present in two bipolar cells. Adjacent Muller cell (M) appears normal and does not contain cytosomes.

The dog was unable to walk or stand and preferred to lie on the floor of the cage at all times. Adult controls were in all respects clinically normal.

**Light microscopy (plastic-embedded 1 μm thick sections).** No obvious differences were seen between CCL and unaffected dog retinas. All retinal layers were present in specimens of affected animals, and there were no obvious cellular loss or abnormality of inner or outer segments.

**Electron microscopy.** Each retinal layer was easily identified in CCL dog specimens. Even at lower magnifications large numbers of abnormal inclusion bodies (hereafter termed "cytosomes") were seen readily within the cytoplasm of retinal neurons and retinal pigmented epithelial cells of all CCL dogs examined (Figs. 1, 3, 5, and 7). With the exception of these cytosomes, all other intracytoplasmic organelles and nuclei appeared entirely normal. The number of such cytosomes was approximately the same in young and adult CCL animals, with no heavier deposition seen in the clinically affected older animals. Muller cells of CCL retinas appeared identical to those in control animals and did not contain the distinctive cytosomes seen in neurons. The morphologic appearance of cytosomes in retinal neurons was different, depending on the cell layer examined. In addition, abnormal material in the retinal pigmented epithelial cells was unique and not seen in retinal neurons. Because of the wide variation in structure of these cytosomes, the following will describe in detail the nature of the abnormal material in cells of each retinal layer.

Ganglion cells contained many irregularly shaped cytosomes which consisted of light, amorphous material and closely stacked collections of membranes resembling finger-
prints (Fig. 1 and 2). Some of the membrane collections appeared as tubular profiles. At higher power individual membranes were spaced 4 to 6 nm apart and consisted of two dark and one light leaflet, each 2 to 2.5 nm in width (Fig. 2). Each cytosome was surrounded by a unit membrane.

Cytosomes within bipolar and amacrine cells of the inner nuclear layer were different from those seen in ganglion cells (Figs. 3 and 4). The background material was much darker and contained arrays of tightly packed, alternating light and dark lines, each 2.5 nm in width (Fig. 4). Usually a membrane with many discontinuities surrounded each cytosome, but on occasion it was entirely absent. On rare occasions cytosomes contained fingerprint profiles identical to those seen in ganglion cells.

In the outer nuclear layer a third type of cytosome was seen; usually in clusters near the nucleus of the cell or in the myoid portion of the cytoplasm (Fig. 5). These membrane-invested cytosomes contained many short, curved profiles. The profiles were faintly osmiophilic and quite difficult to resolve, but appeared to be composed of several alternating light and dark lines, each of which was approximately 2 nm in width (Fig. 6). In addition, some of these cytosomes contained small collections of the same material reported above as occurring in ganglion, bipolar, and amacrine cells.

With the exception of cytosomes in the myoid portion of the inner segment, the inner and outer segments appeared normal. There were no abnormalities of discs in CCL dogs. Contact between terminal outer segments and the retinal pigment epithelial cells appeared the same in CCL and unaffected dogs.

Retinal pigment epithelial cells in CCL dogs of both age groups were filled with the
abnormal, densely staining material (Fig. 7). The most common inclusion was a tightly packed configuration of dark and light lamellae, each 2.5 to 3 nm wide, arranged in linear or curved arrays (Fig. 8). The bulk of this material was freely dispersed, but some appeared to be incorporated into melanin granules (Fig. 8) or into dense bodies (Fig. 9). Normal-appearing phagosomes were present throughout the retinal pigmented epithelium of affected CCL dogs but contained neither lamellar collections or dense bodies (Fig. 9). Other organelles in RPE cells, including mitochondria, rough and smooth endoplasmic reticulum, and nuclei in CCL dogs appeared identical to those seen in normal dogs.

Discussion

This study is the first to our knowledge in which the morphologic abnormalities of CCL dog retina are described. It should be emphasized that although the number of animals reported here is small, similar pathologic and biochemical changes have been seen in subsequent animals examined by us. The pathologic abnormalities in CCL dog retinas are remarkably similar to those reported in brain and spinal cord of these CCL animals.
and it is probable that a common mechanism is operating to produce these changes in various tissues.

In this study the most striking abnormality was the finding of morphologically unique cytosomes within the cytoplasm of retinal neurons. Three different types of cytosomes were seen, and each type was located predominantly in one of three neuronal layers of the retina. Thus, cytosomes with regularly spaced membranes resembling fingerprints constituted the major abnormal deposit in retinal ganglion cells. These collections were similar to those reported in spinal cord ventral horn neurons of CCL dogs, as well as in retinal and cerebral neurons, blood, and thyroid tissues of humans with Batten disease. The predominant cytosome within inner nuclear layer cells contained dark, amorphous material with tightly packed, stacked lamellae coursing through them. These too have been noted in cortical neurons of CCL dogs. The third type of cytosome, most commonly seen in photoreceptor cells, was invested by a prominent membrane and contained short, curved, light and dark lines which were very difficult to resolve adequately. Similar inclusions have been described in thalamic neurons of CCL dogs and bear a striking resemblance to curvilinear profiles reported in retina and brain of Batten disease.

Fig. 6. Higher-power view of cytosomes within photoreceptor cell similar to those in Fig. 5. Each cytosome (arrow) is surrounded by a membrane and contains faintly staining, short, curved profiles suggestive of curvilinear inclusions. Inset, Detail of curved material within cytosome. The inclusions are faint and difficult to resolve but appear to consist of one or more closely packed light and dark lines.
Fig. 7. Retinal pigmented epithelium, 22-month-old CCL dog. The cell cytoplasm contains many abnormal, darkly staining circular and linear profiles (arrows). A normal melanin granule is at right and microvilli (V) appear normal except for postfixation separation from outer segments.

Retinal pigmented epithelial layer were also unique and morphologically different from those seen in retinal neurons. The commonest material consisted of free fragments of multiple tightly packed lamellae in linear or circular arrays. Some of these fragments were incorporated into otherwise normal-appearing melanin granules. These fragments were common in CCL dogs at all ages. The fragments were never found to be joined to or incorporated in any way into phagosomes which, at least by morphologic criteria, appeared entirely normal.

The abnormal cytosomes seen in retinal neurons and retinal pigmented epithelium in our CCL animals are identical, by electron microscopic criteria, to those reported in brain neurons of other CCL dogs. Such cytosomes have been isolated from CCL dog brains and found to contain phospholipids, trace amounts of cations, and a unique acid lipid polymer. This material, termed ceroid by some, is considered to be lipopigment closely related to the aging pigment, lipofuscin. By electron microscopic analysis and chemical composition, ceroid from CCL dog brain and human Batten brains are quite similar. It is likely that ceroid within CCL retina and human Batten retina are also similar, although the small quantities of tissue available to date have prevented such direct chemical assays as done on brain.

At present, the pathogenic mechanism, leading to the formation and accumulation of ceroid lipopigments in retinal neurons and other cells in uncertain. One possibility is that ceroid accumulates in response to increased levels of intracellular peroxides. In the CCL dogs studied here, there is a significantly reduced amount of leukocyte, retinal, and RPE peroxidase in affected dogs. This decrease in peroxidase could
result in increased intracellular peroxide levels which by free radical mechanisms would lead to peroxidation of polyunsaturated fatty acids and formation of ceroid. It might be expected that intracellular mechanisms would operate to remove ceroid and prevent excess accumulation. Recent work on such mechanisms for ceroid removal in neurons and retina of CCL dogs suggests that these functions may be impaired. Thus the combination of low peroxidase causing increased ceroid production and an impaired removal mechanism, possibly related to poor lysosomal response, would lead to a net buildup of ceroid within the retinal neurons and retinal pigmented epithelium.

It is puzzling how the young 6-month-old animals can appear so normal clinically and yet have such massive deposits of ceroid in brain cells. Similarly, in the retina and retinal pigmented epithelium there is massive ceroid buildup but little in the way of visual symptoms until the animals are 1 year of age or older. It has been well established that young 6- to 8-month-old CCL animals, although clinically normal, do demonstrate brain neuron dysfunction in the form of spikewave discharges and delta slowing by electroencephalogram. Preliminary ERG observations on two adult affected dogs have shown a slight reduction of rod b-wave amplitudes. We suggest that such ERG changes could indicate neuronal dysfunction related to the abnormal deposits seen in retinal neurons. The mechanisms underlying these ERG abnormalities could be similar to those producing EEG abnormalities in brain of affected dogs, but larger numbers of animals must be studied to verify this.

The ultrastructural biochemical abnormalities in brain and retina of these CCL dogs have many parallels to those reported in...
human Batten disease. Such comparable abnormalities make the CCL dogs an attractive model for further studies which could be applicable to the human disorder. In terms of retinal changes the deposition of ceroid in CCL retinal neurons is strikingly similar to what is seen in Batten disease.\textsuperscript{4}

One obvious difference between this CCL dog model and human Batten disease is the relatively intact appearance of the photoreceptor cells even in adult animals. In human patients reported by Goebel et al.,\textsuperscript{4} the rod, cone, and outer nuclear layers were markedly attenuated or entirely absent.\textsuperscript{4, 33} Other reports have shown degeneration of the pigment epithelium.\textsuperscript{5-8} In contrast, the CCL animals used in this study showed the outer segments to be normal, the outer nuclear neurons to contain abnormal cytosomes, and the RPE cells to be filled with abnormal membrane profiles. Such total loss of the outer nuclear layer and inner and outer segments in the human patients may be related to the extremely long course of the disease, since all were clinically affected for 8 to 15 years before death. It is possible that if CCL dogs survived longer, comparable damage to these layers would be seen. Unfortunately, the dogs never survive beyond 26 months, which may be insufficient time for morphologic changes to occur in photoreceptor membranes.

There are convincing clinical, pathologic, and biochemical similarities between CCL dogs and patients with human Batten disease. We consider this canine model to be a reasonable one in determining disease mechanisms and studying possible therapeutic approaches. Hopefully, further studies on this model will provide new methods of treatment for the profound retinal and systemic changes occurring in humans.

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