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

I. The distribution of enzymes in the whole retina and pigment epithelium

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The massive accumulation of autofluorescent lipopigments, representative of autoxidation, is a key morphological feature in canine ceroid lipofuscinosis (CCL). In the eye peroxidase, catalase, and four acid hydrolases were compared with regard to age and clinical condition in a series of English setters affected with CCL. In unaffected English setters' soluble peroxidase increased in the RPE to adult levels at 2 yr of age. Affected dogs had higher RPE peroxidase activity earlier in life, which then declined with age. The soluble retinal peroxidase of both unaffected and CCL dogs increased steadily with age, but the latter group of dogs were much lower in activity. By 2 yr of age, RPE and retinal peroxidase values were only 25% and 47% of unaffected dog levels. Although the soluble enzyme of unaffected dogs exhibited a maturational profile, membrane-bound RPE peroxidase showed a hyperbolic curve reaching a maximum at 10 mo of age. By 2 yr of age, the "bound" enzyme in affected dogs was below unaffected levels in the RPE and retina. Three acid hydrolases were slightly increased in the RPE and retina of affected dogs. Acid lipase activity, however, was similar in both unaffected and CCL dogs. Catalase was not found in the RPE of either group of dogs. The catalase activity in the retina of both affected and unaffected dogs was at similar levels. Since catalase is not present in the RPE, the major defense against peroxidase accumulation and peroxide toxicity probably depends upon peroxidase. The present study indicates that a decrease in this key regulating enzyme may be related to the formation of lipopigments in the retina and RPE of dogs with CCL.

Key words: lipopigments, autoxidation, soluble and bound peroxidase, peroxides, compartmentalization, retinal degeneration

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The pathology of degenerative changes in the retina and pigment epithelium (RPE) has been studied in several breeds of dogs. As early as 1911, a group of inbred Gordon set-
Oxidases

\[ \text{O}_2 \rightarrow \text{OH}^+ \]

Diagram 1

Table I. Clinical information on English setters used in the experiments

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Sex</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Female</td>
<td>Unaffected</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>CCL</td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>CCL</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>CCL</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>CCL</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>CCL</td>
</tr>
<tr>
<td>12</td>
<td>Male</td>
<td>Unaffected</td>
</tr>
<tr>
<td>12</td>
<td>Female</td>
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<td>18</td>
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<td>22</td>
<td>Female</td>
<td>Unaffected</td>
</tr>
<tr>
<td>22</td>
<td>Female</td>
<td>CCL</td>
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<tr>
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<td>Female</td>
<td>Unaffected</td>
</tr>
<tr>
<td>24</td>
<td>Female</td>
<td>CCL</td>
</tr>
</tbody>
</table>
Fig. 1. Unaffected dogs (●) show a maturational profile for peroxidase activity whereas, the peroxidase of CCL dogs (△) was seen to peak much earlier in life, followed by a rapid decline in activity.

Fig. 2. Maturational profiles of membrane bound RPE peroxidase in unaffected (●) and CCL dogs (△).

cold (+4° C) glass-distilled water. The sample was centrifuged at 15,000 × g for 15 min, the supernatant was decanted, and this extract was used for determination of the membrane-bound enzyme activity.

In a separate experiment, eyes from four females and five males (all affected), previously frozen for 5 to 7 months, were studied. After being thawed, the retinas were removed from each pair of eyes, pooled, disrupted as were the fresh samples, and assayed.

Soluble RPE peroxidase activity was measured at pH 5, and bound peroxidase at 7.4, with 0.15M phosphate buffer, 20 mM hydrogen peroxidase, and 8.75 mM p-phenylenediamine as the co-substrate. K_m values were previously determined for canine RPE and found to be 8.3 mM for H_2O_2 and 0.32 mM for p-phenylenediamine. One unit of activity was defined as an absorbance change of 1.0 at 485 nm per milligram of protein after 10 min of incubation at 25° C. Both soluble and bound retinal peroxidase were measured at pH 7.4. Catalase was measured by the method of Chance and Maehly. Activity was expressed as a decrease in absorbance at 240 nm per milligram of protein, after 5 min incubation at 25° C. Acid phosphatase, beta-galactosidase, and N-acetyl-β-D-glucosaminidase were measured with their respective 4-meth-
SOLUBLE RETINAL PEROXIDASE, pH 7.4

△ UNAFFECTED
△ CCL
△ FROZEN CCL

AGE (months)

BOUND RETINAL PEROXIDASE, pH 7.4

△ UNAFFECTED
△ CCL

Results

Table I lists the pertinent clinical information on the dogs used in this study, for which both retina and pigment epithelium were simultaneously obtained. The animals, grouped together at each age, were littermates.

The pH optima of soluble and bound peroxidase. Normal soluble RPE peroxidase activity obtained from pooled samples of eyes from pound dogs showed a peak at pH 5 as previously reported.18 The membrane-bound or insoluble enzyme showed a pH maximum at 7.4. The pH optimum for both soluble and bound neural retinas was 7.4. We were not able to do pH curves on affected tissues because we were dealing with only one pair of eyes. However, previous studies on leukocytes from patients with neuronal ceroid lipofuscinosis showed no pH differences from normal.22

Soluble RPE peroxidase activity. Fig. 1 demonstrates the disease- or age-related changes observed for soluble peroxidase. In unaffected English setters, soluble RPE peroxidase increased steadily from 25 units at 6 months of age to an adult level of 240 units at 24 months. In marked contrast, the dogs with CCL showed high soluble activity in the young animals, averaging 383 units in the 6- and 8-month-old dogs and then steadily decreased thereafter to a minimum at 22 to 24 months. At this time, activity was only 25% of that of unaffected controls. The soluble peroxidase in CCL dogs fell below the level of the unaffected English setters within 12 to 13 months.

Insoluble RPE peroxidase activity. Age-related changes of insoluble or membrane-bound peroxidase were quite different from...
those observed at pH 5 for soluble peroxidase (Fig. 2). Bound peroxidase activity was low at 6 months and reached a maximum at 10 months in the unaffected dogs. At this age, bound peroxidase measured at pH 7.4 was approximately three times higher than the soluble peroxidase activity measured at pH 5. The bound peroxidase specific activity then declined and leveled off at an adult level (118 units). The level of bound peroxidase in unaffected English setters 18 to 21 months of age was approximately 50% that of the soluble peroxidase activity. CCL animals showed a similar pattern of bound activity changes with age but were lower in activity at each age group studied.

**Soluble retinal peroxidase activity.** The retinal peroxidase assays were made at pH 7.4 (Fig. 3), as previously reported. The maturational profile of retinal peroxidase in unaffected dogs was similar to that observed in the RPE. However, peroxidase activity was much lower in the retina as compared to the RPE. Thus, depending upon the chronological age at which retinal activity was compared to the RPE, peroxidase was 30 to 150 times higher in the RPE. CCL dogs were similar to unaffected dogs in their activity at 6 months of age, but the affected retinal enzyme appeared to mature more slowly than normal with advancing age. By 2 years of age, retinal peroxidase from CCL dogs was only 47% of the unaffected value.

Retinal peroxidase was determined to be relatively stable when frozen for up to 6 months. In separate experiments we analyzed the soluble peroxidase from nine affected retinas which had been frozen from 5 to 7 months (Fig. 3, black triangles). The activities of the frozen peroxidase were similar to or below the values found in the freshly prepared CCL cases (Fig. 3, clear triangles).

**Bound retinal peroxidase activity.** Bound peroxidase activity (pH 7.4) in the retinas from unaffected dogs also increased with age (Fig. 4) and was three to four times higher than soluble levels (Fig. 3) at 2 years. The bound peroxidase in CCL dogs reached a
maximum much earlier, at 10 months of age and then declined to minimum levels in the adult dogs. At 22 to 23 months of age, the bound retinal peroxidase was only 5.2% of the unaffected value.

**Lysosomal acid hydrolase activities of the RPE.** Because there were no age-related patterns to the four enzymes we studied, values for all ages of unaffected and affected animals were compared directly (Fig. 5). In the RPE of unaffected and affected dogs, N-acetyl-β-D-glucosaminidase showed the highest activity. In both groups of dogs, 89% of the total activity was labile when heated at 50° C for 2 hr, showing that no significant differences were found for the A and B isomer levels of this enzyme. Beta-galactosidase was found in relatively low concentrations, but acid lipase and acid phosphatase were observed at higher levels. With the exception of acid lipase, all lysosomal hydrolases were slightly elevated in the CCL dogs; however, these were not statistically significant.

**Acid hydrolases activities of the retina.** No differences were noted between the hydrolytic enzymes of unaffected or CCL dogs in the retina at any age. N-acetyl-β-D-glucosaminidase was found in the retina at the same specific activity as observed in the RPE (Figs. 5 and 6). This was true for both unaffected and CCL dogs. Again, no significant differences were observed in the isoenzyme levels. After being heated at 50° C for 2 hr, 85% to 89% losses in activity were observed in both groups of dogs. Retinal beta-galactosidase activity was only one-half the specific activity found in the RPE, whereas acid phosphatase was 60% higher in the retina than in the RPE. Acid lipase was very low in the retina.

**Other hydrogen peroxide-degrading enzymes.** Under the usual conditions employed for measuring catalase activity we were never able to demonstrate activity in the purified pigment epithelium of normal pound dogs, nor was catalase activity ever detected in the RPE of unaffected or affected English setters. Catalase activity was present in the retina, but we found no differences in enzyme levels between unaffected and affected dogs.

**Discussion**

The retina is extremely sensitive to damage by in vivo conditions known to produce lipid peroxidation. For example, oxygen and light can easily cause retinal degeneration in animals and man. These same conditions in vitro can result in the peroxidation of numerous membrane constituents through free radical mechanisms. Primarily affected are polyunsaturated fatty acids (PUFA), but in addition, enzymes, amino acids, and mucopolysaccharides can also be damaged.

Peroxidation of lipids containing long-chain PUFA could result from increased intracellular peroxide levels. The present study indicates that changes in levels of peroxidase, a key peroxide-regulating enzyme, may be related and partially responsible for the autofluorescent lipopigments observed in the retinas of dogs with ceroid lipofuscinosis. In characterizing endogenous peroxidase, we have found that a soluble enzyme can be released by sonication and a membrane-bound form released by treatment with 1M salt and detergent. This result was obtained for both neural retina and the pigment epithelium.

At the beginning of our studies, only the report of Berman et al. was available which detailed a technique for isolating RPE cells, and no evidence had been published with regard to the intactness of RPE cells obtained by brushing. Recently, however, Saari et al. have reported that bovine RPE cells are damaged when prepared by the brushing technique, so that almost all (97%) of the binding protein for retinol is released into the first crude RPE cell wash. The authors further indicated that this result was obtained on eyes that were prepared 2 to 3 hr after death. At that time, the bovine RPE cells showed severe damage to plasma membranes. On the other hand, considerably improved RPE preparations were obtained when the procedure was started 15 min after...
death and 0.32M sucrose was used to suspend the cells.

Although we cannot discount the possibility that our canine cells did not incur some degree of membrane damage during the brushing and that diseased cells are more fragile than controls, we nevertheless have employed the best possible conditions for utilizing this technique. Furthermore, to the best of our knowledge, no one has compared the fragility or stability of RPE membranes in the dog prepared by the brushing technique. Obviously, there may be differences between species. For example, we had found, when adapting the technique of Berman et al. that dog RPE cells required only 3 to 4 washes to obtain a pure preparation as compared to the six washes required for bovine cells. Under the light microscope, almost all our dog cells stained dark blue throughout the cytoplasm and were heavily melanized.

In any event, until further work is done to resolve possible differences between species, our soluble fraction should be evaluated in this context. It is conceivable that differences in enzyme localization (soluble vs. bound) between the unaffected and CCL dogs could have been due to the increased fragility of diseased cells. This might result in a loss of enzyme into the wash and lower the enzyme level in our affected animals. Such enzyme loss, however, would most probably be due to cytoplasmic or membrane-bound enzymes. The subcellular localization of enzymes in the RPE has not yet been established. Another finding which would argue against a preferential loss in diseased tissue is the fact that only one enzyme was lower than control values and that was p-phenylenediamine peroxidase. One would certainly expect other enzymes to be similarly lost unless each of the enzymes we studied was localized within different organelles. Berman et al. and Rothman et al. have reported that acid lipase is a cytosol enzyme, and yet we found no differences between unaffected and CCL dogs.

We believe that with these limitations in mind, our studies are still of value in describing the presence of a new enzyme in the RPE and its possible relationship to the process of autoxidation in health and disease.

Considerably more peroxidase activity was found in the pigment epithelium than in the whole retina. In addition to differences between these two tissues, changes in peroxidase levels occurred with age. Thus, unaffected English setters showed a maturation profile of soluble RPE peroxidase reaching adult levels within approximately 2 years of age. On the other hand, CCL-affected English setters demonstrated very high levels of soluble activity at earlier ages, with activity declining below normal levels after just 12 months of age.

When the membrane-bound enzyme fractions from the RPE of unaffected dogs were analyzed, an age-related pattern was observed which was different from that of the soluble enzyme. Membrane-bound peroxidase activity peaked in both unaffected and CCL animals at 10 months of age. During the next 12 to 13 months, the bound enzyme fell steadily, but the decrease of activity in the CCL dogs was much greater.

In the unaffected retina, soluble peroxidase showed a maturation profile similar to that observed for the RPE. The major difference between retina and the RPE was the level of peroxidase; 6.5 units/mg of protein in the retina vs. 218 units in the RPE of adult unaffected dogs. Tissues frozen for up to 6 months showed deficiencies similar to those of fresh tissues, although in three CCL dogs, 13 to 19 months of age, activity was very low. Whether or not this represents the variability seen in the clinical condition of these dogs remains to be ascertained in future studies.

The membrane-bound retinal peroxidase of unaffected animals increased with age. This was similar to the activity-age curve of soluble retinal peroxidase. Changes in membrane-bound enzyme in the CCL retina were different from changes associated with the unaffected enzyme. Thus, as early as 10 months of age, the bound CCL peroxidase had reached its maximum enzyme level. Thereafter, peroxidase activity fell rapidly to extremely low levels. These differences emphasize disease-induced changes which could
account for the retinal degeneration observed in affected animals. This decrease in peroxidase activity cannot be due to just ROS degeneration because (1) photoreceptor segment degeneration is not prominent and (2) we have found in separate studies that peroxidase is not present in the ROS's.

Three lysosomal enzymes were found in the RPE and retina. As seen in most storage diseases, these enzymes were somewhat above normal in the affected RPE and retina in contrast to the decrease in peroxidase which appears unique to this disorder. Acid lipase was barely detectable in the retina but was found in concentrations similar to those of the other hydrolytic enzymes in the RPE. This finding corresponds to the report by Rothman et al.\textsuperscript{34} for bovine eyes, where acid lipase was 30 times higher in the RPE than in the retina. The specific activity reported for bovine acid lipase is, however, 6 times higher than we have seen in dogs. Acid lipase activity in CCL tissues was relatively unchanged from unaffected values. Differences in this enzyme may be a reflection of differences in the lipid composition of the ROS between species. We support the findings of Rothman et al.\textsuperscript{34} that acid lipase is found primarily in the RPE. Other differences between bovine and canine RPE and retinal hydrolytic enzymes are of note. Acid phosphatase, which in the cow is only slightly lower in activity than acid lipase, is more than 80 times higher than acid lipase in the dog. These differences may be reflected somewhat in the different substrates used to measure this enzyme (bovine, $\beta$-glycerophosphate; canine, 4-MU phosphate).

Since catalase was not found in the RPE, the major intracellular defense against peroxide accumulation probably depends on peroxidase. Failure of any enzyme involved with regulating intracellular peroxide or free radical levels could result in oxidative disease. In CCL, a disease where the autofluorescent lipopigments are a major finding, we have demonstrated a progressive deficiency of $p$-phenylenediamine peroxidase in the retina and pigment epithelium. As a result of these altered peroxidase levels, excess hydrogen peroxide could generate hydroxyl free radicals through interaction with superoxide radicals. The unusually high content of docosahexenoic acid\textsuperscript{35, 36} along with the presence of hydrogen peroxide in the ROS would certainly favor lipid hydroperoxide formation. In a process called molecule-assisted homolysis, compounds capable of hydrogen bonding with peroxide groups will weaken the oxygen-oxygen bond and cause decomposition of lipid hydroperoxides.\textsuperscript{37} This results in the generation of additional lipid and peroxyl free radicals. In addition to hydrogen peroxide and hydroxyl free radicals, lipid radicals are well known as cytotoxic agents\textsuperscript{38} and can even destroy rhodopsin.\textsuperscript{39-41} Furthermore, retinal is easily autooxidized and, as such, is inhibitory to lysosomal enzymes such as beta-glucuronidase.\textsuperscript{42}

Superoxide dismutase has been reported in the ROS,\textsuperscript{43} but its over-all distribution in the eye is unknown. Therefore hydrogen peroxide can be generated in the ROS. The outer discs of the ROS will ultimately be shed and phagocytized by the pigment epithelium. Thus peroxides produced within ingested RPE segments are incorporated into RPE phagosomes, there to be degraded by a special form of peroxidase. We propose this mechanism because we have found there to be no detectable catalase and glutathione peroxidase activity in the canine RPE. The pigment epithelium is unlike other phagocytic cells. Because of its low rate of mitosis, these cells should remain in situ for a very long time. Therefore undegradable materials such as peroxidized lipids and rhodopsin would accumulate and could be factors in the pathogenesis of certain retinopathies.

No reports concerning the distribution of the flavin enzymes important in generating superoxide radicals are known to us. It can be presumed, however, that such enzymes do exist in the eye, since superoxide dismutase is present in substantial amounts, at least in the ROS. Thus it is highly probable that, because of the peroxidase deficiency in dogs with ceroid lipofuscinosis, lipid peroxidation occurs at an accelerated rate, and lipopigments are deposited within various cells of
the eye in a progressive and detrimental manner. In support of this statement, we have observed a reduction of electroretinogram amplitude in older CCL dogs. The reason(s) why peroxidase decreases during the progression of CCL is under investigation. Whether this alteration represents some type of inhibition, an alteration of enzyme synthesis, enzyme denaturation, or change in subcellular distribution remains to be determined in future studies.

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REFERENCES

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