In Vitro Glycosylation in the Retina in Canine Ceroid-Lipofuscinosis*

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ABSTRACT

Dolichol-dependent glycoprotein synthesis in the sensory retina of adult dogs with canine ceroid-lipofuscinosis (CCL) has been investigated by studying 3H-mannose uptake in organ culture. Sensory retinal organ cultures were prepared from unaffected littermates and mongrel dogs were used as controls. Morphometric analysis of autoradiographs from dogs without disease showed maximal uptake predominantly into photoreceptors and inner segments, and no abnormalities of incorporation were observed in two affected animals. Tunicamycin blocked the reaction in both samples. Incorporation of 3H-leucine was also normal in CCL retinal tissues. These studies demonstrate that the pathways for addition of mannose residues into dolichol pyrophosphate prior to transfer onto acceptor proteins is similar in normal dogs and those with CCL and does not appear to be the primary lesion in ceroid formation. Its role as a model for lipopigment storage is discussed.

Introduction

Intracellular lipopigments are storage products which exhibit autofluorescence. The age-related lipopigments are referred to as lipofuscin and accumulate in residual bodies. In the human and animal forms of ceroid-lipofuscinosis (CL), the ceroid type of autofluorescence lipopigment accumulates rapidly in childhood and is pathognomonic. Human phenotypes of this syndrome are often referred to collectively by the eponym Batten’s disease, while the two major animal models are identified descriptively as canine and ovine ceroid-lipofuscinosis (CCL and OCL) which denotes that both types of lipopigments are increased in these disorders. Whole homogenates, or purified fractions of lipofuscin and ceroid particles have been analyzed biochemically and contain glycolipids, phospholipids, unsaturated fatty acids, lipid per-
oxides, free radicals, divalent cations, amylloid precursor, adenosine triphosphate (ATP) synthetase proteins and N-linked glycoproteins. Oxidized lipids and proteins, alone or in combination, appear to be capable of forming different fluorophores which impart to lipopigments their characteristic autofluorescent property. An excellent review of current knowledge can be found in a recently published proceedings arising from the fourth international symposium on lipopigments.16

The relationship of polyisoprenols to lipopigment accumulation, especially dolichols, has received considerable interest in recent years since they are known to be concentrated in lysosomal membranes.25 Dolichols are, in fact, also present in high concentration in secondary lysosomes (residual bodies) of aged tissue17 and in tissues from other inborn errors of metabolism where lysosomal proliferation is observed.9 Therefore, measurement of dolichols for the purpose of screening patients with Batten’s disease may be of limited value and their importance as a primary marker for the disease process or in senescence has not been established.

It has previously been reported11 that dolichyl phosphate (Dol-P), but not dolichol (Dol) was elevated in cerebral homogenates from animals with CCL. However, in longitudinal studies, the levels of Dol-P were actually normal up to 3 months of age, when the number of ceroid particles is appreciable.

The present study has investigated the formation of N-linked glycoproteins in tissues from the normal and diseased neural retina of the English setter model studied in culture. Dol-P is a carbohydrate intermediate in the synthesis of glycoprotein. In the presence of endogenous acceptor, radiolabelled mannose is coupled to the terminal N-acetylglactosamine and the resulting oligosaccharide incorporated into membrane protein through N-linked glycosylation. The locations of glycan-containing mannose acceptors within the retina are reported and the relevance of abnormal dolichol metabolism in CL is discussed.

Materials and Methods

BUTTON EXPLANT CULTURES

Animal experiments conformed to the provided guidelines.* Eyes were obtained from mongrel dogs, a normal English setter at age 42 months, and two animals with CCL at age 15 and 25 months. The anterior segment of each eye was dissected away, the vitreous expressed and full-thickness retinal specimens removed with a three mm trephine.21 Tissues were placed directly into Ringers bicarbonate-glucose media, containing 50 μCi (for light microscopy) or 600 μCi (for electron microscopy) of D-[2,3H]-mannose per ml of media, (54 mCi per mmol) or 4 μCi (56 mCi per mmol) of L-[4,5-3H]-leucine,† one percent dimethylsulfoxide (DMSO), gassed with 95 percent O₂-5 percent CO₂ and incubated in a Dubnoff Metabolic Incubator for 3.5 hours at 37°C. Samples were also incubated in media containing tunicamycin (20 μg per ml) which inhibits N-acetyl glucosamine-1-phosphate transfer of dolichyl phosphate from UDP-glucosamine and thus blocks formation of lipid oligosaccharides and N-linked glycoproteins.6

Autoradiography

Light Microscopy. Tissues were washed in four changes of medium and then fixed in two percent glutaraldehyde for studying radiolabelled mannose

* Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, 1985.
† Amersham Corp.
incorporation, or in two percent formalin followed by two percent glutaraldehyde for the leucine incorporation experiments. All tissues were post-fixed for one hour at 4°C in one percent osmium tetroxide, dehydrated with 2.4-dimethoxypropane, cleared with propylene oxide and embedded in Epon-Araldite. Sections (1 μm) were coated with Kodak Nuclear Track (NTB-2) emulsion, placed in the dark for one month, developed in Kodak Dektol Developer (1:1 aqueous), and stained with toluidine blue for histological examination. The autoradiograms were photographed under dark-field illumination on a Nikon Labophot microscope equipped with an AUFx automatic exposure meter.

**Electron Microscopy.** Additional sections of 800 to 900Å thickness were stained with uranyl acetate and lead citrate, coated with Ilford Nuclear research (L-4) emulsion, placed in the dark for four months, and developed in Ilford, Phenidone developing agent. The autoradiograms were photographed on a Zeiss 9 transmission electron microscope.

**Morphometrics**

Silver grains were counted over 12 equal areas in three locations across the retina. Three specimens were analyzed per eye cup. Counts were entered into an Apple IIe computer for plotting of the data.

**Results**

Histological examination of embedded sections showed good preservation of structure when compared to unincubated, fresh tissue. Radiolabel was present in all cells of the normal and CCL retina, but was highest within the photoreceptor inner segment and outer nuclear cell layers (figures 1 and 2). Some ganglion cells incorporated mannose while others apparently did not, and a small amount was observed in the outer segment layer. Very little label was found in the extracellular space.

Even though pathology was present in the affected animals, the distribution pattern of 3H-mannose into rods or cones was of the same magnitude as observed for the normal control and the dogs with CCL (figures 3 and 4). Tunicamycin was effective and inhibited the incorporation of mannose in our system (figure 5).

Explants incubated with 3H-leucine exhibited a greater degree of incorporation per unit area than did mannose and a more uniform distribution was found over all cell layers in the retina (figures 6 and 7). As observed in the mannose experiments, only a few grains were seen in the region of the outer segments.

Morphometric analysis of grain counts taken from sections incubated with mannose showed a definite gradient from the inner to outer regions of the retina (figure 8). One-third of all silver grains were located in the photo-receptor cells of the outer nuclear layer. In control and CCL retinal tissue, another 35 percent of the label was incorporated within the inner segments, while only three percent was present in the outer segments. The remaining label was rather uniformly distributed across the inner nuclear and ganglion cell layers of the retina. Grain counts from the affected animals were not statistically different from control specimens.

**Discussion**

The most consistent pathological feature of the ceroid-lipofuscinoses is the progressive accumulation of autofluorescent lipopigment. A similar phenomenon is also seen in aged tissues from mammalian and invertebrate sources. These chromophoric inclusions, whether ceroid or lipofuscin, are considered to originate through a series of oxidative
events, possibly initiated by a free radical attack on polyunsaturated lipids with the formation of lipid peroxides, followed by metal catalyzed degradation which liberate reactive carbonyls to participate in Schiff base cross-linking, polymerization, or protease inactivation. Ceroid has been shown to interfere with retinal function when a critical intracellular level is achieved and is cytotoxic in its mature form.

The present studies began by attempting to measure Dol and Dol-P in the retina directly, but since tissue extracts from a single retina was insufficient in mass for detection by HPLC, it was elected to measure de novo synthesis of lipid-linked oligosaccharide glycoproteins in retinal explants via the reaction of $^3$H-mannose using endogenous acceptors. Such an experiment basically reflects mannosyltransferase activity and indicates the integrity of regulatory status in these tissues.

Wolfe et al have reported elevated levels of retinoic acid-like material in ceroid isolated from human CL patients at autopsy. Later, this group identified dolichol as the major constituent of the aqueous phase, and subsequently reported high levels in the urine of patients with ceroid lipofuscinosis.
However, dolichol also accumulates with age in the normal human brain\textsuperscript{17} and elevated levels are also associated with other metabolic disorders.\textsuperscript{9} The specificity of dolichol to the disease process in ceroid-lipofuscinosis is therefore unclear. It has been previously reported that in CCL brain, no elevation of dolichol occurs, even in end-state disease when maximum deposition of ceroid is present.\textsuperscript{11} However Dol-P, the physiological form was found to be elevated, but only after six months of age when there is already considerable storage of...
In the present study, it has been demonstrated that \(^{3}\text{H}\)-mannose is taken up primarily by rod and cone photoreceptor cells and their inner segments. In vitro studies by Fliesler et al\(^5\) have also shown that mannose incorporates rapidly and preferentially (85 to 90 percent) into photoreceptors of human retinal explants and is highest in the inner segments. These authors found that rods contained approximately twice as many counts as did cones. Our results in canine tissues tend to show an equal distribution among rods and cones in normal, heterozygote or homozygote dogs with CCL. In none of the retinal layers was any difference observed in the incorporation pattern.

The pivotal role of Dol-P in N-linked glycoprotein synthesis has previously been defined.\(^{13}\) Keller et al have recently

ceroid. An increase of Dol-P in human ceroid-lipofuscinosis has also been observed. Evidence suggesting that Dol-P elevation might represent a secondary event in CCL, was obtained by demonstrating that dolichol phosphatase, the regulatory enzyme for these lipids, was within the normal range.\(^{11}\)
quantified the levels of Dol and Dol-P in normal frog retina, while Anderson et al have shown that ROS degeneration occurs when dolichol synthesis is inhibited in vivo using tunicamycin. Furthermore, in the rds mouse model, a mutant gene encoding for N-linked glycosylation of an essential ROS membrane-specific 39 kd protein, leads to progressive degeneration of ROS. Therefore, measurement of glycoprotein synthesis provides a sensitive index regarding retinal pathology. Our results suggest that dolichol-linked reactions leading to the incorporation of mannose into retinal membrane glycoproteins is normal in CCL. Protein synthesis, using leucine as a probe, was also normal in CCL samples. Thus, these metabolic pathways appear intact in a disorder where storage of autofluorescent lipopigments are markedly increased. This accelerated model of aging should continue to be a valuable resource in understanding how lipopigment correlates with morphological and biochemical change in senescent tissue.

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