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Retinal Degeneration in a Rat Model of Smith-Lemli-Opitz Syndrome: Thinking Beyond Cholesterol Deficiency

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Abstract

Smith-Lemli-Opitz Syndrome (SLOS) is a recessive hereditary disease caused by a defect in the last step in cholesterol biosynthesis— the reduction of the $\Delta 7$ double bond of 7-dehydrocholesterol (7DHC)— resulting in the abnormal accumulation of 7DHC and diminished levels of Chol in all bodily tissues. Treatment of rats with AY9944— a drug that inhibits the same enzyme that is genetically defective in SLOS (*i.e.*, DHCR7, 3β -hydroxysterol- $\Delta 7$ -reductase)— starting *in utero* and continuing throughout postnatal life, provides a convenient animal model of SLOS for understanding the disease mechanism and also for testing the efficacy of therapeutic intervention strategies. Herein, the biochemical, morphological, and electrophysiological hallmarks of retinal degeneration in this animal model are reviewed. A high-cholesterol diet partially ameliorates the associated visual function deficits, but not the morphological degeneration. Recent studies using this model suggest that the disease mechanism in SLOS goes well beyond the initial cholesterol pathway defect, including global metabolic alterations, lipid and protein oxidation, and differential expression of hundreds of genes in multiple ontological gene families. These findings may have significant implications with regard to developing more optimal therapeutic interventions for managing SLOS patients.

1.1 Introduction

Cholesterol is a ubiquitous lipid constituent in all mammalian cells and tissues. In the plasma membrane of most higher eukaryotic cells, cholesterol typically accounts for 30–50 mol % of the total lipid (reviewed in: Yeagle 1985). It is no mere quirk of fate that cholesterol, as opposed to literally thousands of other possible sterols, is the overwhelmingly dominant, if not sole, resident sterol in such cells and tissues. Other sterols do not seem to provide the essential features requisite to promote and preserve normal cellular structure, function, and viability (reviewed in: Demel and DeKruyff 1976; Bloch 1989). Perhaps the most clear-cut evidence for the essential role that cholesterol plays in human biology is the existence of a group of devastating, often lethal, hereditary human diseases that involve specific, genetically determined defects in the enzymes responsible for cholesterol biosynthesis (reviewed in: Porter 2003). While each of these diseases has a distinct phenotype, they all involve dysmorphologies as well as profound defects in the development and function of the nervous system. The first and best described of these diseases, as well as the most common, is Smith-Lemli-Opitz Syndrome (SLOS) (Smith, Lemli and Opitz 1964; reviewed in: Porter 2008). The primary biochemical defect in SLOS involves the enzyme DHCR7 (3β -hydroxysterol- $\Delta 7$ -reductase; EC1.3.1.21), which converts 7-dehydrocholesterol (7DHC) to cholesterol (Fig.1) (reviewed in: Correa-Cerro and Porter 2005). Over a hundred disease-causing mutations in the DHCR7 gene, spread throughout its 9 exons, have been

discovered (reviewed in: Yu and Patel 2005), and the spectrum of disease severity is quite broad, from mild to lethal.

Despite decades of study, the biological roles of cholesterol in the retina have yet to be fully elucidated. In the course of our studies of cholesterol biosynthesis and metabolism in the retina (reviewed in: Fliesler and Keller 1997; Fliesler 2002), we asked two fundamental questions: 1) What are the consequences of depleting the retina of its endogenous cholesterol, with regard to its development, structure, and function; and 2) Can sterols other than cholesterol support the normal development, structure and function of the retina? Given the information provided above, we reasoned that disrupting cholesterol biosynthesis should have significant and deleterious consequences on retinal development, histological and ultrastructural organization, and electrophysiological function. Herein, we show that these expectations were born out, using a pharmacologically induced rat model of SLOS.

1.2 The AY9944 Rat Model of SLOS: Biochemical Findings

Studies performed in the 1960s (reviewed in: Gofflot 2002) demonstrated that treatment of rodents (mice and rats) with inhibitors of cholesterol biosynthesis had profound effects on embryogenesis and early postnatal development. One of these inhibitors was AY9944 (*trans*-1,4-bis(2-chlorobenaminomethyl)cyclohexane dihydrochloride), a relatively selective inhibitor of DHCR7 (Dvornik et al 1963; Givner and Dvornik D 1965), the same enzyme as is defective in SLOS (see Fig. 1). With the elucidation in 1993–94 of the link between defective cholesterol biosynthesis and SLOS, it became evident that treatment of rodents with such inhibitors could yield a model of SLOS (Xu et al 1995; Kolf-Clauw et al 1996). Typically, however, those rodent models tended not to be viable for more than a few days to a week.

Since the neural retina in rats and mice develops over the course of the first four postnatal weeks, we refined and optimized the conditions and dosage of administering AY9944 in Sprague-Dawley rats to yield a SLOS model that reproduced the biochemical hallmarks of the human disease (*i.e.*, markedly elevated 7DHC levels and reduced cholesterol levels in all tissues) while also maintaining viability for at least three postnatal months (Fliesler et al 1999, 2004, 2007). Fig. 2 shows an example of a typical reverse-phase HPLC chromatogram of nonsaponifiable lipids extracted from the retinas of one-month-old AY9944-treated (Fig. 2A) and control rats (Fig. 2B). In this case, the 7DHC/cholesterol mole ratio in the treated animal's retina was almost 4:1, whereas in the control it's zero, since 7DHC is not normally present at appreciable steady-state levels in the retina. The rats are maintained on a cholesterol-free diet, such that their only source of sterols is that derived biogenically via *de novo* synthesis. The key to improved viability is not to expose the fetuses to AY9944 within the first gestational week, to provide a low dosage of AY9944 during gestation, either by feeding the pregnant dams chow containing 1 mg AY9944 per 100 g chow (Fliesler et al 1999, 2004) or by continuously infusing them with a sterile, PBS-buffered solution of AY9944 (0.37 mg/kg/day; at 2.5 microL/h) via an Alzet® osmotic pump (Fliesler et al 2007), and injecting the pups (typically three times per week) subcutaneously with buffered AY9944 solution (25–30 mg/kg). Under the conditions of our studies, the 7DHC/cholesterol mole ratio typically is >5:1 in both serum and liver within the first postnatal month of exposure to AY9944, and can reach values of >11:1 by three months postnatal. This biochemical hallmark exceeds that of even the most severely affected SLOS patients (Tint et al 1995). In the case of the retina, by three months of AY9944 treatment the 7DHC/cholesterol mole ratio is typically >5:1; hence, there is a progressive increase in the relative amount of 7DHC in retina, from one to three months.

1.3 Retinal Degeneration in the SLOS Rat Model: Histology and Ultrastructure

The histological changes in the retina that occur as a function of postnatal age in the AY9944-induced SLOS rat model have been presented in detail elsewhere (Fliesler et al 1999; Fliesler et al 2004) and are summarized in Fig. 3. About the first postnatal day (P1) the cells of the retina, with the exception of the ganglion cell layer (GCL), are still not yet differentiated and the retina of AY9944-treated rats (Fig. 3A) appears virtually identical to that of an age-matched control (not shown). Subsequently, up to about the first postnatal month, the retina undergoes maturation and the histological organization and appearance of the retina in AY9944-treated rats (P33, Fig. 3B) again is essentially equivalent to those of age-matched control rats (not shown). However, by about the second postnatal month, the number of pyknotic nuclei in the outer nuclear layer (ONL; *i.e.*, photoreceptors) dramatically increases, and the thickness of the ONL and photoreceptor outer segment (OS) layer is noticeably reduced in AY9944-treated rats (P69, Fig. 3C), relative to age-matched controls (not shown). By 10–12 postnatal weeks, the ONL and POS layer show further, marked reductions ((P93, Fig. 3D). Quantitative morphometric analysis (Fliesler et al 2004) has shown that, on average, the OS layer thickness is reduced by nearly a third (30–35%) while ONL thickness is reduced by 20–25%, relative to controls, by three postnatal months of treatment, and the degeneration is fairly symmetrical across the vertical meridian.

Ultrastructurally, rod outer segments in the SLOS rat model, while shortened relative to age-matched controls after one postnatal month (Fliesler et al 1999), appear otherwise normal, even after three postnatal months when retinal degeneration has progressed significantly (Fliesler et al 2004). However, the RPE is decidedly abnormal, compared to controls, by three postnatal months in AY9944-treated rats (Fig. 4). The RPE cytoplasm becomes grossly congested with phagosomes (ingested tips of shed outer segment membranes), lipid droplets, and a variety of membranous inclusion bodies, regardless of what time of day tissue specimens are prepared. [Normally, such material is largely cleared from the RPE cytoplasm within the first 1–2 hours after light onset in animals maintained in cyclic light.] That said, the polarity of the RPE cells appears relatively normal, with well-extended apical villi and mitochondria lined up proximal to the basal infoldings of the RPE plasma membrane. Given the large load of ingested ROS tips and accumulation of phagosomes in the SLOS rat RPE, one might expect the A2E levels to be substantially elevated, relative to controls. However, preliminary studies (J.R. Sparrow and S.J. Fliesler, unpublished) indicate this is not the case. Regardless, given the pathological condition of the RPE and the severely shortened ROS in this model, one would predict that visual function should be substantially compromised. That turns out to be true (see below).

1.4 Retinal Degeneration in the SLOS Rat Model: Electrophysiological Deficits

Within the first postnatal month, retinal function in the SLOS rat model is robust, with scotopic (rod) ERG amplitudes being at least as great, if not greater than, those of age-matched controls, although the implicit times for the scotopic b-waves are greater (response timing more sluggish) than in controls (Fliesler et al 1999). However, by three months of AY9944 treatment, both rod and cone ERG amplitudes are significantly reduced and photoresponse timing is delayed, compared to controls (Fliesler et al 2004). In addition, both rod sensitivity (*S*) and maximal photoresponse (R_{mP3}) values are reduced about two-fold, relative to age-matched controls. These findings correlate well with the histological and ultrastructural observations (see above), and indicate that there is progressive visual dysfunction in the SLOS rat model, both with regard to phototransduction efficiency as well

as postreceptor signal transmission. Importantly, these findings are consistent with the rod (Elias et al 2003) and cone (A.B. Fulton, personal communication) functional deficits observed in SLOS patients.

1.5 Effects of Feeding a High-Cholesterol Diet

Interestingly, feeding a high (2%, by wt.) cholesterol diet can yield marked improvements particularly in the cone ERG responses (Fliesler et al 2007): photopic b-wave amplitudes are increased nearly two-fold, approaching normal levels, and b-wave timing is also significantly improved (although still not normal). The effects of the high cholesterol diet on the rod system, however, are far less pronounced, although there is substantial improvement in both a- and b-wave implicit times. Notably, dietary cholesterol supplementation in the SLOS rat model actually results in near normalization of the steady-state levels of cholesterol in the retina by three postnatal months, while also significantly lowering the 7DHC levels (by about 30%), such that the 7DHC/cholesterol mole ratio is reduced by three-fold, compared to age-matched SLOS rats fed cholesterol-free chow (Fliesler et al 2007). Despite this, however, histological degeneration of the retina is not spared, although there is a substantial (and statistically significant) reduction in the number of pyknotic photoreceptor nuclei in the retinas of cholesterol-fed SLOS rats compared to those maintained on a cholesterol-free diet.

1.6 Perspective: Thinking Beyond the Cholesterol Deficiency in SLOS

While the standard of care for managing SLOS patients is cholesterol supplementation therapy (reviewed in: Porter 2008), the efficacy of this approach is quite variable and, typically, minimal. Indeed, the above findings suggest that while restoring cholesterol levels in the retina is helpful, there's something missing in the treatment regimen that prevents full rescue of retinal structure and visual function. We propose that, although cholesterol deficiency caused by a defect in DHCR7 is the *primary* (initiating) cause of the disease, SLOS involves *secondary* defects in non-sterol metabolic pathways as well as additional biochemical changes.

Using the SLOS rat model, we've shown that there is a dramatic and progressive loss of docosahexaenoic acid (DHA, 22:6n-3) in whole retinas (Ford et al 2008) and ROS membranes (Battaglia et al 2008), compared to age-matched controls. As expected, such dramatic alterations in membrane fatty acid composition cause profound perturbations in ROS membrane fluidity (Battaglia et al 2008), which undoubtedly reduces the efficiency of phototransduction (as reflected in the scotopic a-wave parameters). The presence of lipid hydroperoxides (LPO) in SLOS rat retinas, at levels comparable to those observed in photodamaged rats, has been demonstrated (Richards et al 2006), and the LPO levels increase dramatically when SLOS rats are exposed to intense constant light, correlating with the extent of histological damage observed in the retina under such conditions (Vaughan et al 2006). Furthermore, preliminary reports have indicated the oxidative modification of retinal proteins with reactive aldehydes that are end-products derived from oxidative degradation of both n-3 and n-6 fatty acids (Fliesler et al 2006). Such protein modifications are known to compromise protein structure and function and have been implicated in various diseases that involve oxidative stress (reviewed in Negre-Salvayre A 2008). Curiously, rhodopsin (the overwhelmingly dominant ROS membrane protein) somehow evades such modifications (Fliesler et al 2006). In addition, initial DNA microarray analysis has revealed that hundreds of genes are differentially expressed in retinas of SLOS rats, compared to age-matched controls (Siddiqui et al 2007, 2008), with the number and types of differentially expressed genes correlating well with the time course of retinal degeneration. Notably,

among the affected gene families are those associated with oxidative stress and regulation of cell death.

Given the evidence for oxidation of both lipids and proteins in the SLOS rat model, in addition to the expected sterol pathway modifications, we suggest that future development of therapeutic interventions for clinical management of SLOS patients should include antioxidants in addition to cholesterol supplementation. Determining which antioxidants to use, the dosage, timing, and formulation with cholesterol will be challenging. The SLOS rat model described herein offers an excellent test system with which to evaluate the efficacy of these various factors in advance of randomized clinical testing in human patients.

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Fig. 1.1.

The cholesterol pathway, indicating the defective biochemical reaction in Smith-Lemli-Opitz Syndrome (SLOS), normally catalyzed by DHCR7 (3 β -hydroxysterol- Δ^7 -reductase), and the site at which AY9944 inhibits the pathway. The chemical structures of cholesterol and 7-dehydrocholesterol (7DHC) are shown. FPP, farnesylpyrophosphate; $\Delta^{5,7,24}$, 7-dehydrodesmosterol.

Fig. 1.2.

Reverse-phase HPLC chromatogram of retinal nonsaponifiable lipids from a one-month old AY9944-treated rat (*left panel*) and an age-matched control rat (*right panel*). Note the dominance of 7DHC and the markedly lower level of cholesterol (denoted by the asterisk *) in the treated sample, whereas cholesterol is the only detectable sterol in the control. Detection by UV absorbance at 205 nm (A205).

Fig. 1.3. Histology of the retina in the SLOS (AY9944-treated) rat model as a function of postnatal development. (A) Postnatal day (P) 1; (B) P33; (C) P69; (D) P92. Arrows (*panel C*) indicate pyknotic nuclei in the photoreceptor layer. Note progressive thinning of the outer segment (OS) layer and outer nuclear layer (ONL) with age. GCL, ganglion cell layer; INL, inner nuclear layer; IS, inner segment layer; RPE, retinal pigment epithelium.

Fig. 1.4.

Ultrastructure of the neural retina and RPE in a three-month old control rat (A) and in an age-matched SLOS rat (B). Note the substantial congestion of the RPE with phagosomes (denoted by asterisk *) and other densely-staining membranous inclusions in the SLOS rat retina, compared to the control. The general ultrastructural appearance of rod outer segments (ROS) in both panels is comparable. Chor, choroid.