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Alterations of the *Tunica Vasculosa Lentis* in the Rat Model of Retinopathy of Prematurity

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Abstract

Purpose—To study the relation between retinal and *tunica vasculosa lentis* (TVL) disease in ROP. Although the clinical hallmark of retinopathy of prematurity (ROP) is abnormal retinal blood vessels, the vessels of the anterior segment, including the TVL, are also altered.

Methods—ROP was induced in Long Evans pigmented and Sprague-Dawley albino rats; room-air-reared (RAR) rats served as controls. Then, fluorescein angiographic images of the TVL and retinal vessels were serially obtained with a scanning laser ophthalmoscope (SLO) near the height of retinal vascular disease, ~20 days-of-age, and again at 30 and 64 days-of-age. Additionally, electroretinograms (ERGs) were obtained prior to the first imaging session. The TVL images were analyzed for percent coverage of the posterior lens. The tortuosity of the retinal arterioles was determined using Retinal Image multiScale Analysis (RISA; Gelman *et al.*, 2005).

Results—In the youngest ROP rats, the TVL was dense, while in RAR rats, it was relatively sparse. By 30 days, the TVL in RAR rats had almost fully regressed, while in ROP rats it was still pronounced. By the final test age, the TVL had completely regressed in both ROP and RAR rats. In parallel, the tortuous retinal arterioles in ROP rats resolved with increasing age. ERG components indicating postreceptor dysfunction, the *b*-wave and oscillatory potentials (OPs), were attenuated in ROP rats.

Conclusions—These findings underscore the retinal vascular abnormalities and, for the first time, show abnormal anterior segment vasculature in the rat model of ROP. There is delayed regression of the TVL in the rat model of ROP. This demonstrates that ROP is a disease of the whole eye.

Introduction

The clinical hallmark of retinopathy of prematurity (ROP) is abnormal retinal blood vessels but, in fact, ROP is a disease of the whole eye. For instance, infants born prematurely, especially those with ROP, are at increased risk for developing a range of structural ophthalmic sequelae including impaired ocular growth and increased incidence and magnitude of refractive error, particularly myopia [1, 2]. Myopia is a mismatch between the

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light-focusing power of the anterior segment and the axial length of the eye in which the visual image comes to a focus in front of the retina. Myopia is, therefore, typically associated with longer-than-average eyes [3]. Paradoxically, in ROP myopia the eye is often short [1, 2, 4, 5]; the refractive power of the lens and cornea must be higher than normal. In addition, visual impairment, with a basis in the neural retina, is commonly found in subjects with a history of ROP, even when the vasculopathy was mild [6, 7], and an increasingly large body of evidence indicates that the appearance of the abnormal retinal blood vessels in ROP is actually instigated by changes in the neural retina [8, 9]. Thus, these common ROP outcomes – vascular, structural, and neural – are likely interrelated.

In common with the vessels of the posterior eye, the vessels of the anterior segment are abnormal in ROP. The severity of these abnormalities is associated with the severity of the retinal disease [10]. Persistent and dilated iridic vessels are known to be prognostic of progression to severe ROP as defined by the retinal vessels [10]. The *tunica vasculosa lentis* (TVL), the part of the hyaloid that supplies the developing lens, normally regresses by term but is present preterm [11, 12] and persists, much engorged, in severe ROP [12, 13]. Changes to the TVL may affect development of the anterior segment and contribute to the broad distribution of refractive errors in ROP that is well-documented [14, 15].

Rat pups exposed to alternating relatively high and relatively low oxygen during the first weeks after birth develop a retinopathy that models human ROP [16, 17]. This oxygen-induced retinopathy represents a convenient *in vivo* model in which to study ROP that has been widely adopted, the so-called “ROP rat.” The ROP rat's vascular abnormalities include an avascular peripheral retina and neovascularization [18-20], as in human ROP [21]. The original ROP rat was the albino Sprague-Dawley. However, recent studies suggest that retinopathy in the pigmented rat may be just as, if not more, severe [22, 23]. Also as in human ROP, retinal function is persistently abnormal [24-33]. Furthermore, while the regression of the rat hyaloid is normally well coordinated with the development of other ocular structures, such as the vitreous chamber and crystalline lens, in the ROP rat, growth of ocular structures and hyaloidal regression proceed in a relatively uncontrolled manner [34]. Image analysis was used to investigate the development of the retinal and TVL vessels and the electroretinogram (ERG) to study retinal function in the ROP rat, albino and pigmented. The inclusion of pigmented animals provided increased fluorescein contrast against the background choroidal vasculature. The hypothesis that retinal vascular and neural disease would be associated with increased hyperemia of the TVL was tested.

Methods

Subjects

Seven Long Evans pigmented and six Sprague-Dawley albino rats were studied. All animals were born in Boston Children's Hospital. There, three Long Evans and three Sprague-Dawley rats had retinopathy induced following the Penn *et al.* “50/10” paradigm [18] as previously described [25, 27, 31, 35]. In brief, to induce retinopathy, newborn rat pups were exposed to alternating 24 hour periods of 50% and 10% oxygen starting on the day of birth and lasting for two weeks, when they were returned to room air (~21% oxygen). Littermates were room-air-reared (RAR) and served as controls. The rats, along with their nursing dam, were shipped overnight for study at the Centre for Ophthalmology at the University of Tübingen, Germany. There, ERGs were obtained and the animals were imaged at 19-22 days-of-age (~20), 30 days-of-age, and 64 days-of-age, as described below. During the course of the study, one Long Evans ROP rat was lost after the first examination, and one Long Evans RAR was lost after the examination at 30 days. The institutional animal care and use committees at each respective institution approved these experiments.

Electroretinography

In the ~20-day-old rats, immediately prior to imaging, ERGs were recorded binocularly as described previously [36]. Rats were dark-adapted overnight and anesthetized (at 64 days-of-age, 87.5 mg/kg ketamine and 12.5 mg/kg xylazine; at younger ages, 46.8 mg/kg ketamine and 9.3 mg/kg xylazine). Gold-wire ring electrodes were placed on the cornea, and silver needle electrodes served as reference (forehead) and ground (tail). The ERG apparatus consisted of a Ganzfeld bowl, a DC amplifier, and a PC-based control and recording unit (Multiner Vision; VIASYS Healthcare GmbH, Hoechberg, Germany). The pupils were dilated with tropicamide eye drops (0.5%; Mydriaticum Stulln, Pharma Stulln, Germany). Flash ERGs were elicited from the dark-adapted eyes using stimuli ranging from 10^{-4} to $25 \text{ cd}\cdot\text{s}\cdot\text{m}^{-2}$, in ten increasingly bright steps. Ten responses were averaged at each stimulus level. Band-pass filter cut-off frequencies were 0.3 and 300 Hz.

The activation of phototransduction was characterized by fit of the Hood and Birch [37] formulation of the Lamb and Pugh [38, 39] model to responses at the two highest intensity levels. The key parameter extracted from this model, Rm_{p3} (μV), describes the saturated amplitude of the rod photoreceptor response. Postreceptor cell function, Rm_{p2} (μV), was calculated from the saturating bipolar cell response [40]. Inner retinal responses (*e.g.*, amacrine and ganglion cells) were evaluated from the saturating energy in the oscillatory potentials (OPs), Em ($\mu\text{V}^2\cdot\text{s}^{-1}$) [29]; the square root of OP energy ($Em^{1/2}$) was used in the final analysis as it is proportional to summed OP amplitude (μV). All response parameters were respectively derived from both eyes and then averaged to produce a single value for each animal.

In vivo imaging

Scanning-laser ophthalmoscopy (SLO) images were obtained from anesthetized rats (immediately after ERG recordings, at ~20 days), as described previously, using a Heidelberg Retina Angiograph (HRA I; Heidelberg Engineering, Heidelberg, Germany) [45]. The HRA I features two visible argon wavelengths (488 nm/514 nm) and two infrared diode lasers (795 nm/830 nm). To image the retinal vessels and TVL, we performed fluorescein angiography (FLA) at three time points using the 488 nm laser stimulus with a barrier filter at 500 nm. Fluorescein-Na solution (University Pharmacy; University of Tübingen, Germany) was injected subcutaneously for rats smaller than 120 g and intravenously for rats larger than 120 g. Fluorescein-Na dosing was 12.5 mg for rats weighing less than 50 g, 25–50 mg for rats weighing 50–120 g, or 2.5 mg for rats weighing more than 120 g. The images were obtained with the confocal plane in two respective positions: at the retinal surface to capture an ~20° field of the ocular fundus (centered on the optic disk), or at the back of the lens to capture the TVL.

Tortuosity of the retinal arterioles (T_A) was evaluated in the FLA angiograms using Retinal Image multiScale Analysis (RISA) software as previously described [27, 31, 41]. Briefly, each vessel was cropped from the main image and segmented individually. RISA constructed a skeleton and marked terminal and bifurcation points. The operator then selected the vessel segments for analysis and RISA automatically calculated the integrated curvature for the selected segments of each vessel. Arteriolar tortuosity, T_A (radians·pixel⁻¹), was calculated for each subject as the mean integrated curvature of all arterioles in each eye (median 6). TVL coverage was measured using the ‘Trainable Segmentation’ plugin [42] in Fiji [43]. The operator identified a few regions of the image as corresponding to background and vessels, and the computer automatically segmented all pixels containing vasculature. The operator then bounded the lens to circumscribe a region of interest (ROI) and Fiji computed the ratio of vascular to total pixels (so that 0% would be a completely avascular

lens and 100% would be a lens completely covered by TVL). A sample segmentation is shown in Figure 1.

Analyses

All RISA, TVL, and ERG parameters were expressed as log values, and individual values were then normalized to the mean value in the RAR rats ($\log$ RAR). Statistical tests are described in the relevant section of the Results.

Results

In the ROP eyes, as shown in a representative set of images from a Long Evans rat (Figure 2), the tortuosity of the retinal arterioles was higher at young ages and decreased with age. Simultaneously, the TVL regressed between 22 and 30 days-of-age and was no longer detectable by 64 days-of-age. Figure 3 displays the retinal vessels and TVL in an age-matched RAR eye. T_A was lower in RAR rats than in ROP rats and did not change much throughout the developmental period studied. As in ROP, TVL coverage regressed between 22 and 30 days-of-age to become undetectable by 64 days-of-age, but coverage was *much* less in RAR than ROP rats. Indeed, in the Long Evans rats in particular, TVL coverage in ROP eyes was so great that it was an average of 0.5 log units higher at age 30 days (Figure 2) than in RAR eyes at age 22 days (Figure 3), despite the eight additional days for regression to occur in the ROP rat.

ERGs were also clearly altered in ROP eyes. Figure 4 shows mean $\pm$ SD ERGs obtained at 0.0010, 1.0, and 25 cd $\cdot$ s $\cdot$ m $^{-2}$ from the ROP and RAR Long Evans rats. Particularly, postreceptoral components such as the *b*-wave and oscillatory potentials were found to be reduced in ROP eyes, whereas direct photoreceptor contributions (such as the *a*-wave in saturation) appeared not to be substantially affected.

Figure 5 plots key vascular and ERG parameters (in $\log$ RAR units) obtained in $\sim$ 20-day-old ROP and RAR, Long Evans and Sprague-Dawley rats. Group (ROP vs. RAR) by strain (Long Evans vs. Sprague-Dawley) ANOVA on T_A (*panel A*) indicated that the increase in tortuosity in ROP was significant ($F_{\text{group}}=12.5$; $df=1,9$; $p=0.0063$). TVL coverage (*panel B*) likewise was significantly greater in ROP rats than RAR rats ($F_{\text{group}}=66.4$; $df=1,9$; $p=1.9\times 10^{-5}$). A group by strain by parameter (Rmp_3 , Rmp_2 , $Em^{1/2}$; *Panels C-E*) repeated measures ANOVA detected that ROP rats had reduced retinal function ($F_{\text{group}}=69.6$; $df=1,9$; $p=1.6\times 10^{-5}$); notably, the amplitudes of inner retinal (postreceptor) responses (Rmp_2 , $Em^{1/2}$) were increasingly more affected by ROP than photoreceptor responses ($F_{\text{group}\times\text{parameter}}=130$; $df=,18$; $p=2.0\times 10^{-11}$). In *panel F*, mean $\pm$ SEM values for rod photoreceptor (Rmp_3), bipolar cell, (Rmp_2) and inner retinal ($Em^{1/2}$) response amplitudes are plotted.

From the data obtained in the $\sim$ 20 day-old ROP rats, the parameters T_A , Rmp_3 , Rmp_2 , and $Em^{1/2}$ were plotted as a function of TVL coverage and inspected for significant monotonic relationships using Spearman's rank correlation (ρ) statistical test. T_A and TVL coverage showed a perfect, positive rank correlation ($\rho=1$) with a nearly linear $\log T_A$ vs. $\log$ TVL product moment correlation ($r^2=0.90$, slope=0.42). $Em^{1/2}$ was also significantly associated with TVL ($\rho=-0.87$; $p=0.019$). These results are consistent with anterior blood vessels, retinal blood vessels, and neural retinal function being mutually affected by ROP.

Discussion

Despite the fact that ROP is clinically characterized by abnormalities in the retinal vasculature, dilated and engorged hyaloidal and iridic vasculature are also conspicuous in

ROP. The ROP rat is well documented to display increased retinal vascular tortuosity, and herein we show that this occurs in parallel with an exuberant vascularization of the TVL.

The tortuosity of the retinal arterioles resolved from ~20 days-of-age (near the height of the vascular abnormalities) to adulthood, during which time the TVL completely regressed. In cross-sectional data, a one-to-one correlation between retinal vessel tortuosity and TVL lens coverage in ROP rats evidenced a linkage between these parameters. With respect to the increased tortuosity of the retinal arterioles: It may be a direct consequence of the reduced size of the vascular bed; like the meanders of a river, tortuosity may be an attempt to reduce flow velocity, and thus increase perfusion. With respect to the TVL: Reduced perfusion causes the retina to release a variety of compensatory factors, such as VEGF. Perhaps, increased levels of such vasoactive factors lead, in turn, to persistence of vitreous vasculature, including profuse TVL. Consistent with earlier studies showing greater abnormality in pigmented than albino ROP rats [22, 23], both retinal vessel tortuosity and TVL lens coverage were greater in the Long Evans than Sprague-Dawley rats. Furthermore, the strong association with inner retinal function ($Em^{1/2}$) suggests that these same mechanisms may also pattern neural development. The retinal vasculature and the postreceptor neural retina are in close physical proximity, are immature at the same ages, and develop together. Indeed, we have identified interrelations between postreceptor retinal sensitivity, retinal vascular abnormality (T_A), and mRNA expression of the growth factors VEGF₁₆₄ and Sema3A [31], which are known to cooperatively control both angiogenesis and neurogenesis [44, 45].

In our studies of human ROP patients, we are confronted repeatedly with these issues: Even when the vascular disease is mild and resolves without intervention, ROP eyes are at greatly heightened risk for persistent visual dysfunction that is attributable to the retina, arrested eye growth, and high refractive error, especially myopia. In the ROP eye, it is plausible that anterior segment development would be profoundly altered by this anomalous, persistent vascularization. For instance, the thicker lens that has been documented in the biometry of ROP eyes [46, 47] may be one consequence. The paradoxical myopia, attributed in part to the thicker lens, might therefore be secondary, in part, to the altered TVL. Since the ROP rat's lens contributes approximately two thirds of the refracting power of its eye [48], it is an attractive system in which to study the role of the lens in ROP myopia.

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Figure 1.

Sample evaluation of the *tunica vasculosa lentis* (TVL) coverage of the lens. **A)** Fluorescence angiographic image of the TVL in a 22-day-old Long Evans ROP rat obtained by scanning laser ophthalmoscopy (SLO). **B)** The regions of the SLO image identified as vessels by the ‘Trainable Segmentation’ plugin [42] in Fiji [43] are highlighted (*red*) and the region of interest (ROI) is indicated (*yellow dashes*). **C)** The cropped region of interest is segmented into vasculature (*red pixels*) and background (*white pixels*). The ratio of red-to-white pixels was used to calculate percent TVL coverage of the lens.

Figure 2.

Scanning laser ophthalmoscope (SLO) fluorescein angiographs of the retinal surface (*top*) and the back of the lens (*bottom*) in a ROP Long Evans rat at the postnatal days (P) indicated. The proportion of adult normal tortuosity of each retinal arteriole is displayed (*red boxes*) and the average tortuosity for all arterioles at each age, T_A , is displayed below the respective image. T_A decreased steadily from P22 to P64. The proportion of the TVL covering the lens is shown for the first two ages. TVL imaging was unsuccessful in adult rats.

Figure 3.

2. Scanning laser ophthalmoscope (SLO) fluorescein angiographs of the retinal surface (*top*) and the back of the lens (*bottom*) in a RAR Long Evans rat at the postnatal days (P) indicated. The proportion of adult normal tortuosity of each retinal arteriole is displayed (*blue boxes*) and the average tortuosity for all arterioles at each age, T_A , is displayed below the respective image. T_A was very stable throughout development, and much lower than in age matched ROP rats (Figure 2). The proportion of the TVL covering the lens is shown for the first two ages. By P30, the TVL nearly completely regressed; note that while the retinal surface vessels are visible in the background of the P30 lens image, there is only a wisp of lens vasculature in the foreground.

Figure 4.

Averaged responses (*lines*) and one SD (*fills*) from the 22-day-old Long Evans ROP (*red*) and the RAR (*blue*) rats for three stimuli selected from the dark-adapted ERG intensity series. The dim flash (*bottom*) evoked a marked *b*-wave in the RAR rats, but – in the absence of a discernible *a*-wave – almost no response in the ROP rats. The moderate flash (*middle*) evoked a pronounced *a*-wave in both the ROP and RAR rats, but elicited a much-attenuated postreceptor response (*b*-wave and OPs) in the ROP rats. The bright flash (*top*) evoked a saturating *a*-wave in both groups. In the RAR rats, the *a*-wave was large, the OPs were pronounced and the *b*-wave rose well above baseline; in the ROP rats, both the *b*-wave and the OPs were strikingly enervated but the *a*-wave was relatively preserved.

Figure 5.

Log individual (*symbols*) and mean (*line*) parameter values in RAR (*blue*) and ROP (*red*) Sprague-Dawley (*open circles*) and Long Evans (*filled triangles*) rats plotted as the proportion normal (mean of all RAR rats) are shown in *panels A-E* for tortuosity of the arterioles (*panel A*), TVL coverage of the lens (*panel B*), amplitude of the saturated rod photoreceptor response (*panel C*), amplitude of the saturated bipolar cell response (*panel D*), and amplitude (square root of energy) in the saturated oscillatory potentials (OPs; *panel E*). In *panel F*, response parameters from both strains in photoreceptor (Rm_{P3}), bipolar (Rm_{P2}) and inner retinal cells ($Em^{1/2}$) show that higher order responses are more attenuated in ROP than RAR rats.