Crumbs homolog 1 (CRB1) mutations result in a thick human retina with abnormal lamination

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Received February 3, 2003; Revised February 24, 2003; Accepted March 2, 2003

Mutations in CRB1, the human homolog of Drosophila Crumbs, cause autosomal recessive blinding disorders of the retina. Whereas Crumbs is implicated in apical-basal epithelial polarity and photoreceptor morphogenesis, the role of CRB1 in normal or diseased retina remains unclear. We characterized the retinal organization in vivo of patients with CRB1 mutations and found that, unlike other inherited retinal degenerations studied to date, the CRB1 mutant retinas are remarkably thick in cross-section and lack the distinct layers of normal adult retina. There are coarse outer and inner zones and a thick surface layer around the optic nerve. The abnormal retinal architecture in CRB1 mutations resembles that of immature normal retina. The results suggest that the CRB1 disease pathway disturbs the development of normal human retinal organization by interrupting naturally occurring apoptosis.

INTRODUCTION

Crumbs (Crb) was the first Drosophila gene shown to be a key developmental regulator of epithelial apical-basal polarity (1). The transmembrane protein Crb is now known to be one of many protein complexes along the plasma membrane that participate in cell-to-cell contacts, such as zonulae adherentes. The molecular interactions of Crb with other proteins controlling epithelial cell polarity in Drosophila are actively being explored (2,3). CRB1, one of three Crb human homologues (4), came to scientific and medical attention when mutations in this gene were identified in severe forms of retinal blindness known as retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA) (5–7).

The sequence from CRB1 mutation to the human phenotype of congenital retinal blindness seemed more understandable when a role in retinal photoreceptor morphogenesis was demonstrated for Drosophila Crb and murine Crb1 (8,9). CRB1 would be expected to be important for human photoreceptor function and structure (8,9) and Crb and CRB1 were suggested to have conserved functions in eye development (4). Mutations in CRB1 certainly lead to loss of photoreceptor signaling by clinical electrophysiological testing in affected patients (7), but so do all other known genetic causes of RP and LCA (10). Little further detail about the pathogenesis of human CRB1 mutation has been forthcoming and there is currently no mammalian disease model to gain insight into the detailed mechanism.

Here we used in vivo high-resolution microscopy (11) to study the retinas of patients with CRB1 mutations. In contrast to the phenotypes of other inherited retinal degenerations studied to date by histopathology (12) or in vivo microscopy (13,14), CRB1 mutant retinas were abnormally thick rather than thinned and the lamination pattern was coarse, resembling certain stages of normal human retinal development (15–17). These unexpected results in CRB1 mutations may be due in part to a disturbance in retinal histogenesis with interrupted naturally occurring apoptosis.

RESULTS

The CRB1 mutant human retina viewed en face (Fig. 1A, inset) appears abnormal due to pigment clumping (18). This surface view cannot define pathological features in depth because the retina is transparent. We thus used high-resolution

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cross-sectional retinal imaging, known as optical coherence
tomography (OCT) (11,19–21), to study the in vivo
micro-
pathology of LCA due to CRB1 mutations and compared the
results to LCA from other molecular causes (10).

Normal human central retina viewed with OCT has a foveal
depression and discernible layers (Fig. 1A, upper part). A
CRB1 mutant retina (Table 1; patient 6) also has a foveal
depression, but the surrounding retina appears thicker than
normal with less distinct lamination (Fig. 1A, lower part). We
quantified central retinal thickness (Fig. 1B) in eight CRB1-
LCA patients (ages 2–50; Table 1) and related these data to
normal (n = 18; ages 4–29). CRB1 mutant retinas have a foveal
depression but the adjacent retina is abnormally thick. To
determine if this result is CRB1-specific, we analysed retinal
thickness in LCA due to mutations in RPE65, a retinal pigment
epithelium (RPE) gene involved in retinoid metabolism, and
GUCY2D, a photoreceptor gene controlling phototransduction
recovery (Table 1; patients 9–11). These patients have normal
or reduced retinal thickness (Fig. 1B), like other retinal
degenerations (12–14).

When we examined the laminar organization of CRB1 mutant
retinas, we found that the thick retinas were not simply
expanded versions of normal. Normal human retinal lamination
is shown in a histological section from the temporal retina of a
53-year-old man (Fig. 2A, left). There are three cellular layers
(ganglion cell layer; inner nuclear layer; outer nuclear layer);
two synaptic layers (inner and outer plexiform layers); the inner
limiting membrane (the basal lamina of Muller glia); photo-
receptor inner and outer segments; the outer limiting membrane
(zonulae adherentes that join photoreceptor inner segments to
Muller glia); and the RPE. The nerve fiber layer is not visible at
this locus along the horizontal raphe. An OCT retinal cross-
section from a normal 58-year-old man is shown at the same
temporal location (Fig. 2A, right). Bands of low and high
reflectivity are discernible: cellular layers generally correspond
to low reflectivity OCT bands and synaptic layers have higher
reflectivity. Photoreceptor, RPE and anterior choroid form the
highly reflective layer deep in the retina (20,21).

Reflectivity waveforms, which comprise the OCT scans, show
the differences in retinal layering between normals and LCA in
temporal retina (Fig. 2B) and nasal to the fovea near the optic
nerve (Fig. 2C). An average normal (n = 3, ages 19–25)
waveform from temporal retina (Fig. 2B) has peaks and troughs

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**Table 1. Mutations in patients with LCA**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Patient/gender/age (years)</th>
<th>Amino acid change(s)</th>
<th>Reference</th>
</tr>
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<tr>
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<tr>
<td></td>
<td>4/F/18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Arg764Cys</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>5/F/24</td>
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<tr>
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<td>6/M/26</td>
<td>749del3bp</td>
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<td>7</td>
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<td>Lys801Stop</td>
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<td>Tyr368His</td>
<td>42&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Arg660Gln&lt;sup&gt;d&lt;/sup&gt;</td>
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</table>

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<sup>a</sup>All patients are unrelated with the exception of 4 and 5, who are siblings.

<sup>b</sup>Clinical diagnosis may also be considered as early-onset severe RP.

<sup>c</sup>Mutation previously reported by other authors.

<sup>d</sup>STRP indicates deletion in complementary allele.
corresponding to the light and dark bands in Figure 2A. RPE65 and GUCY2D mutant retinas are thinned mainly due to loss of outer nuclear layer and photoreceptor inner and outer segment components. These waveforms resemble those in other human and animal retinal degenerations with photoreceptor loss (13,14,20,21). In contrast, the reflectivity waveforms from CRB1 mutant retinas are about 1.5 times thicker than normal (Figs 1B and 2B) and the pattern does not relate easily to normal layering. We defined three reflectivity zones: β, an inner zone of high reflectivity; γ, an adjacent deeper zone with lower reflectivity; and δ, the deepest zone which, like the normal photoreceptor-RPE-choroidal complex, is highest in reflectivity.
Nerve fiber layer thickness normally increases toward the optic nerve as ganglion cell axons from across the retina converge to exit the eye. By OCT, the nerve fiber layer is a band of high reflectivity near the retinal surface (Fig. 1A). Overlaid reflectivity waveforms from nasal and temporal retina in normal subjects illustrate the additional surface reflection (Fig. 2C, left), which relates well to nerve fiber layer thickness by histology (22). The CRB1 mutant retina also has an additional surface reflection present nasally but not temporally in overlaid waveform templates (Fig. 2C, right). This component in CRB1, labeled \( \alpha \), is far thicker than normal. The spatial distribution of \( \alpha \) (Fig. 2D) within an annular region around the optic nerve (inset) in a CRB1 mutant retina (Table 1; patient 3), compared with normal nerve fiber layer \( (n = 4; \text{ages} 19–25) \), illustrates the pronounced thickening in CRB1. Polar plots of nerve fiber layer thickness at a single radius (Fig. 2E) around the optic nerve (inset) in five CRB1 eyes (patients 2–6) confirm that the LCA inner retinal layer \( \beta \) is indeed thicker than normal nerve fiber layer \( (n = 24; \text{ages} 8–41) \). Histological nerve fiber layer measurements at four loci in normal human eyes (22) fell within our normal range by OCT measurement (Fig. 2E).

**DISCUSSION**

This is the first report of coarsely-laminated thick retinas in LCA or any human genetic retinal disease. The opposite, retinal thinning, is usually observed and was described recently by histopathology in LCA due to \( \text{RPE65} \) by \( \text{GUCY2D} \) (23) mutations, consistent with our \textit{in vivo} results in this study. Normal human fetal retina, however, is thick and coarsely laminated (15–17). For example, at about 24 weeks of gestation, there can be a foveal depression but only 'primordial' laminar formation in more peripheral retina, 1.5-fold numbers of ganglion cells and 2-fold optic axon numbers, compared with the adult (15–17,25,26). Also, thick retinas with increased neurons and synapses are features of mice with disturbed naturally occurring apoptosis during development, such as \( \text{bcl}-2 \) overexpressing transgenic and \( \text{bax}/-/- \) mice (reviewed in 27).

We hypothesize that the CRB1 mutant retinal phenotype represents an immature laminar pattern resulting from interrupted naturally occurring developmental apoptosis. The broad inner retinal zone \( \beta \) would be a layer with increased ganglion cells among synaptic and glial elements. The higher reflectivity \( (\alpha) \) around the optic nerve would represent excess ganglion cell axons \( (16,17) \). The deeper low reflectivity \( (\gamma) \) would be an undeveloped nuclear zone \( (16,17) \) and the deepest high reflectivity \( (\delta) \) and backscatter would be abnormal photoreceptor/RPE layers, possibly with photoreceptor rosettes.

Understanding the causal pathways leading to this previously unrecognized retinal phenotype in LCA will require considerable further work on the precise role of CRB1 during human retinal development. Murine \( \text{Crb}1 \) is considered to be important for development of photoreceptor cells, most notably in zonula adherens assembly (4,8,9). Zonulae adherentes form the outer limiting membrane, the site of interconnection of photoreceptor inner segments and Muller glia. Dysplasia mainly at the photoreceptor level can result from outer limiting membrane disruption and Muller glia abnormalities (28,29), but the type of lamination defects that extend from outer to innermost retina, such as we found in CRB1 mutations, has not been described. It would be intriguing if the abnormal CRB1 retinal organization is the consequence of perturbed communication from neuron-to-glia-to-neuron (specifically ganglion cells, the radially-oriented Muller glia and photoreceptors), a signaling pathway proving to be important in vertebrate and invertebrate retinal laminar development (30). From a clinical perspective, it will be valuable to determine how these structural defects affect retinal circuitry and possibly central visual pathways. We could then assess the relevance to patients with CRB1 mutations of therapeutic strategies emerging for other LCA genotypes (31).

**MATERIALS AND METHODS**

Subjects: clinical and molecular analyses

Informed consent for all procedures was obtained from subjects after the nature of the studies had been explained. Research procedures were in accordance with institutional guidelines and the tenets of the Declaration of Helsinki. Blood samples were obtained and DNA extracted (32). Probandns were screened for mutations in \( \text{CRB1, RPE65} \) and \( \text{GUCY2D} \) using single-strand conformation polymorphism analysis (SSCP). The primer sequences used have been reported (5,7,33,34). The polymerase chain reaction (PCR) amplification products were denatured for 3 min at 94°C and then electrophoresed on 6% polyacrylamide–5% glycerol gels at 25 W for about 3 h. The gels were stained with silver nitrate (35). PCR products showing shifts were sequenced bidirectionally using an automated sequencer (model 377; Applied Biosystems, Foster City, CA, USA).

Table 1 shows participating patients \( (n = 11) \) and their gene mutations. Subjects with normal eye examinations \( (n = 26; \text{ages} 4–58 \text{ years}) \) were included as controls for the various studies. All patients had severe retinopathy that falls within the spectrum of LCA. Two of the eight patients with CRB1 mutations (Table 1; patients 3 and 4), however, showed better visual acuity \( (20/40 \text{ and } 20/60, \text{respectively}) \) than the others (range, 20/100 to light perception). An alternative diagnosis for these two patients could be early-onset severe RP. The patterns of inheritance in the CRB1 patient group were autosomal recessive \( (n = 1) \), multiplex \( (n = 3) \) and simplex \( (n = 4) \). Most of the patients were hyperopic and had nystagmus. All CRB1 patients had limited kinetic visual fields, mainly showing severely impaired central and far peripheral islands only. Electroretinography indicated no detectable responses to standard stimuli except in patients 1 and 3, who had measurable but abnormally reduced waveform amplitudes. Patient 8 had keratoconus and had undergone bilateral corneal transplants years before his visit. Most patients had pigmented abnormalities across the fundus and attenuated retinal vessels. Optic nerve drusen were not present. Three patients showed preserved para-arteriolar retinal pigment epithelium and one had Coats' disease (18,36).

**Optical coherence tomography: data acquisition**

Cross-sectional retinal reflectivity profiles were obtained with optical coherence tomography (OCT; Zeiss Humphrey...
Instruments, Dublin, CA, USA). The principles of the method (19,37) and our techniques (13,14,20,21) have been published. For the current work, data were acquired on two types of instruments: a first-generation OCT1 with a theoretical axial resolution in retinal tissue of ~10 μm and a third-generation OCT3 with a resolution of ~8 μm. All patients and all normal subjects were scanned with OCT1; in addition, six patients with CRB1 mutations as well as a subset of normal subjects were scanned with OCT3. Three types of scan groups were used to sample the retinal cross-section across the posterior pole. One scan group consisted of five overlapping segments of linear scans, each of 4.5 mm length, located along the horizontal meridian, centered on the anatomical fovea and extending to 9 mm in either direction. The second scan group consisted of six linear scans, each of 4 mm length, in a regular spoke pattern (with 30° increments) centered on the optic nerve. The third group was three concentric circles of 1.8, 3.6 and 5.4 mm diameter, also centered on the optic nerve. In addition, a circular scan of 3.4 mm diameter and a horizontal linear scan of 4.5 mm length were acquired centered on the optic nerve. Each type and location of scan was repeated at least twice and usually many times. A video fundus image was acquired and saved with each OCT scan by the commercial software. In addition, the fundus video visible during the complete session was recorded continuously on a video cassette recorder. This allowed post-acquisition analysis of possible eye movements occurring during a scan (see below).

Many of the patients displayed involuntary eye movements (nystagmus) which complicated the recording of OCT scans. In most cases, the saccadic amplitudes of the nystagmus varied over time during the session. To obtain OCT scans, we waited for near-steady periods, judged by looking at the real-time fundus view. In cases with more severe nystagmus, video records from the session were analysed post-acquisition and data from scans obtained during saccades were discarded. This accounts for partial retinal thickness data displayed in Figure 1B for three of eight patients with CRB1 mutation and the patient with GUCY2D mutation.

OCT: data processing and quantification

Post-acquisition processing of OCT data was mostly performed with custom programs (MATLAB 6.5, MathWorks, Natick, MA, USA). OCT cross-sectional data are presented either on a pseudocolor scale (see bar in Fig. 1A) or a gray scale; displays have the vitreous above. For magnified images (see right panel in Fig. 2A), a 5 x 1 moving median filter was used to smooth speckle noise in lateral dimension while keeping the original high resolution in the longitudinal direction. For quantitative analysis, the precise location and orientation of each scan relative to retinal features (blood vessels and optic nerve head) were determined using the video images of the fundus. The individual reflectivity waveforms (Fig. 2B and C) were allotted to 0.3 mm bins in a rectangular coordinate system centered at the fovea; the waveforms in each bin were aligned using a dynamic cross-correlation algorithm (20,21), normalized and averaged. The retinal thickness, as measured by OCT, was assumed to be the distance between features T1 (corresponding to the vitreo-retinal interface) and T5 (corresponding to the maximal slope scleral to the signal maximum, as previously defined in 20,21,38). Reflectivity waveform templates from a group of individuals (Fig. 2B and C) were produced by first scaling each waveform to the average T1–T5 distance of the respective group and then averaging the scaled waveforms. This preserved both quantitative thickness measures as well as waveform shape.

Thickness of the nerve fiber layer was calculated with the commercial programs included in each OCT instrument. In short, the commercial software uses the depth of the high reflectivity signal near the vitreo-retinal interface as an accepted measure of the thickness of nerve fiber layer around the optic nerve region (11,37,39). For each of the 12 scans around the optic nerve (six concentric scans, six linear scans in a spoke pattern), nerve fiber layer thickness was determined as a function of scan position. A custom program (MATLAB 6.5) was used to sample (using bilinear interpolation) the non-uniformly spaced nerve fiber layer thickness data from these 12 scans onto a uniform (30 μm) square grid in a 6 x 6 mm region centered at the optic nerve. The resulting data were spatially filtered with a moving average filter (300 x 300 μm in extent) five times to smooth out noise, mapped to a pseudocolor scale and presented as a floating ‘surface’ illuminated by two point light sources. In addition, the nerve fiber layer thickness determined from the 3.4 mm diameter ‘standard’ circular scans was presented as a polar plot where the location around the optic nerve defined the angle and the thickness defined the radial length of each datum. All data were displayed as equivalent right eyes.

Retinal histology

An eye from a 53-year-old man with normal vision was obtained after surgical exenteration for an orbital tumor. The eye was opened and fixed immediately in 4% paraformaldehyde in 0.1 M phosphate buffer. The central retina containing the macula and optic nerve head was dissected and infiltrated overnight in 30% sucrose in the same buffer at 4°C. The tissue was cryosectioned at 12 μm thickness and processed for immunofluorescence with a cone specific antibody (mAb 7G6, 1:250, from Dr P. MacLeish, Morehouse School of Medicine, Atlanta, GA, USA). Secondary anti-mouse IgG (1:50) was labeled (red) with Cy-3 (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). Cell nuclei were stained (blue) with 4',6'-diamidino-2-phenylindole (1 μg/ml; Molecular Probes, Eugene, OR, USA). Control sections were treated in the same way with omission of primary antibody. The immunolabeled retinal sections were imaged with a microscope equipped for epifluorescence (Leica DMR, Deerfield, IL, USA).

ACKNOWLEDGEMENTS

We thank J. Emmons, M. Kulon, Y. Huang, I. Knizhnik, J. Chico, A. Cheung, M. Nguyen, K. Przychodzen, E. Windsor, A. Pantelyat, J. Smith and M. Swider for critical help. V.C.S. is an associate investigator and E.M.S. is an investigator of the Howard Hughes Medical Institute. This work was supported by the National Institutes of Health EY13385, EY13729, EY13203, Foundation Fighting Blindness, Daniel Matzkin Research Fund and Macula Vision Research Foundation.
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