Localization of Tubby-like Protein 1 in Developing and Adult Human Retinas

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PURPOSE. To localize tubby-like protein 1 (TULP1) in developing and adult human retinas.

METHODS. TULP1 was localized by immunofluorescence microscopy in human retinas, aged 8.4 fetal weeks to adult. TULP1-positive cells were identified by double label with antibodies specific for cones, rods, and astrocytes.

RESULTS. In adult retinas, anti-TULP1 labels cone and rod inner segments, somata, and synapses; outer segments are TULP1-negative. A few inner nuclear and ganglion cells are weakly TULP1-positive. In fetal retinas, cells at the outer retinal border are TULP1-positive at 8.4 weeks. At 11 weeks, the differentiating central cones are strongly TULP1-reactive and some are positive for blue cone opsin. At 15.4 weeks, all central cones are strongly positive for TULP1 and many are reactive for red/green cone opsin. At 17.4 weeks, central rods are weakly TULP-reactive. In peripheral retina at 15.4 weeks to 1 month after birth, displaced cones in the nerve fiber layer are positive for TULP1, recoverin, and blue cone opsin. Some ganglion cells are weakly reactive for TULP1 at 11 weeks and later, but astrocytes and the optic nerve are TULP1-negative at all ages examined.

CONCLUSIONS. The finding of TULP1 labeling of cones before they are reactive for blue or red/green cone opsin suggests an important role for TULP1 in development. TULP1 expression in both developing and mature cones and rods is consistent with a primary photoreceptor defect in retinitis pigmentosa (RP) caused by TULP1 mutations. Weak TULP1-immunolabeling of some inner retinal neurons in developing and adult retinas suggests that optic disc changes in patients with RP who have TULP1 mutations may be primary as well as secondary to photoreceptor degeneration. (Invest Ophthalmol Vis Sci. 2000;41:2352–2356)

In tubby mice, also known as retinal degeneration 5 (rd5), a recessive mutation in the tub gene causes progressive retinal and cochlear degeneration and adult-onset obesity with insulin resistance.1,2 The mutation in the tub gene that leads to these phenotypic alterations is a G→T transversion that abolishes a donor splice site, leading to replacement of the carboxyl-terminal 44 amino acids with a 20-amino acid sequence not found in the wild-type protein.3 Tub is a member of a small neuronally expressed gene family that includes human TUB (homologous to mouse tub), tubby-like protein 1 (TULP1), TULP2, and TULP3. The tubby proteins have a highly conserved carboxyl terminal region but divergent amino terminals, and related proteins are found in lower animals and plants. The TUB gene is expressed in multiple human tissues including retina, whereas the TULP1 gene product is found mainly in retina. TULP2 is expressed primarily in testis,3 and TULP3 is found in multiple tissues, including the retina.5,6

Recent evidence suggests that TULP1 is a transcription factor involved in control of downstream genes in retinal photoreceptors.7 Autosomal recessive retinitis pigmentosa (RP) develops in patients homozygous for mutations in the TULP1 gene.8–11 A recent report of a large family in the Dominican Republic described the phenotype of patients homozygous for a splice site mutation (IVS14 + 1, G→A) in the conserved carboxyl region of TULP1 gene.11 In the first decade of life, the affected persons have nystagmus, absent rod function, and severely impaired cone function throughout the retina. The early vision loss is suggested to result from photoreceptor maldevelopment, dysfunction, or degeneration.11

The Dominican Republic family members with RP also show early optic disc abnormalities suggestive of degenerative changes in the ganglion cells and/or the optic nerve. One study of mouse retina found tub expression restricted to ganglion cells during embryogenesis and mainly in photoreceptors in postnatal and adult animals.12 A more recent study found tub expressed in both ganglion cells and photoreceptors in adult mouse retina.10 The clinical findings, together with the tub expression pattern in retina, raises the question of whether TULP1 is expressed not only in photoreceptors but also in ganglion cells and the optic nerve in developing human retinas.11 Recent studies9,15 demonstrate TULP1-immunolabeling of mouse photoreceptors. However, cones are a minor population in mouse retina, and we wanted to know whether...
human cones also express this protein. To resolve these questions and provide needed information on the pathophysiology of retinal degeneration caused by TULP1 mutations, we used immunocytochemistry to localize the TULP1 protein in developing and adult human retinas.

METHODS

Tissue Preparation

Adult human eyes were obtained through the donor programs of the Foundation Fighting Blindness (Hunt Valley, MD) and the University of Washington Lions Eye Bank (Seattle). Fetal human eyes were obtained from the University of Washington Human Embryology Laboratory. The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all donors. The research was approved by the institutional human subjects review boards of the University of Washington and the University of Pennsylvania. Eight normal adult retinas were evaluated, all fixed at 6 hours or less after death, along with retinas fixed within 1 hour after death at fetal ages 8.4, 9, 11, 14, 15.4, 17.4, 18, 19, and 21.5 weeks and postnatal ages 3 days and 1 month. The globes were fixed for several weeks to months in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), and stored thereafter in 2% paraformaldehyde for several weeks to months in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), and stored thereafter in 2% paraformaldehyde in the same buffer.

Immunocytochemistry

Retinal samples were cryosectioned at 12 μm and processed for immunofluorescence according to published techniques.14 The secondary antibodies (goat anti-rabbit or anti-mouse IgG) were labeled with fluorescein isothiocyanate (green), Cy-2 (green), or Cy-3 (red; Jackson ImmunoResearch, West Grove, PA). Nuclei were stained with DAPI (1 μg/mL; Molecular Probes, Eugene, OR). Control sections were treated in the same way with omission of primary antibody.

The anti-TULP1 (TULP1-N) was a rat polyclonal antibody (pAb) against the amino-terminal half of human TULP1,15 used at a dilution of 1:750 to 1:1000. Cell-specific antibodies prepared in rabbits or mice were used for double labeling to analyze the various retinal cell types labeled with anti-TULP1. Rods were identified with anti-rhodopsin, a mouse monoclonal antibody (mAb; 4D2; 1:40; from Robert Molday, University of British Columbia, Vancouver, Canada). Cones were marked with rabbit pAb anti-blue cone opsins (JH455; 1:5000–1:10,000) and anti-red/green cone opsins (JH492; 1:5000–1:10,000; from Jeremy Nathans, Johns Hopkins University, Baltimore, MD). Cones were also labeled with rabbit pAb anti-red/green cone opsins (1:200; from John Saari, University of Washington, Seattle). The rods, cones, and flat midget bipolar cells were identified with rabbit pAb anti-recoverin (1:1000; from Alexander Dizhoor, Wayne State University, Detroit, MI). Astrocytes were labeled with pAb anti-glial fibrillary acidic protein (GFAP; 1:500; Dako, Carpinteria, CA).

Immunolabeled retinal sections were photographed with an epifluorescence microscope (DMR; Leica, Deerfield, IL) using Elite Chrome film (ASA 400; Eastman Kodak, Rochester, NY). Images were digitized with a flatbed scanner (HiRes; Saphir, Heidelberg, Germany) with Elite 5.1 software (LinoColor, Heidelberg, Germany) and imported into a graphics program (Photoshop 5.0; Adobe, San Jose, CA) from which dye-sublimation prints were generated.

RESULTS

Adult Human Retinas

Strong TULP1-immunolabeling was present in rod inner segments, somata, and synapses (Fig. 1A). TULP1-reactivity was present but somewhat less intense in the cone inner segments, whereas cone somata and synapses were heavily labeled (Fig. 1A). No TULP1-labeling was found in rod or cone outer segments (Fig. 1A). Double labeling with anti-TULP1 and anti-recoverin demonstrated absence of TULP1-reactivity in the photoreceptor outer segments but colocalization of both proteins in the cone and rod inner segments, somata, and synapses (Figs. 1B, 1C). A few cells in the inner nuclear and ganglion cell layers were weakly TULP1-positive (Figs. 1A, 1D). Control sections treated with only Cy3-labeled secondary showed autofluorescence of lipofuscin in the retinal pigment epithelium (Fig. 1E).

Fetal Human Retinas

Because of the steep developmental gradients in early fetal retinas,15 sections were studied that were cut along the horizontal meridian in or near the fovea. All retinal neurons showed a central to peripheral gradient of maturation, and the fetal retinas showed a central to peripheral appearance of TULP1-immunolabeling. At the earliest ages examined, 8.4 and 11 fetal weeks, TULP1-labeling was found in the outermost cells of the central retina lying opposite the retinal pigment epithelium (RPE; Fig. 1F). The remainder of the neuroblasts, stained intensely blue with DAPI, were weakly TULP1-positive (Fig. 1F).

By 21.5 weeks, the neuroblast layer in the periphery was much thicker and TULP1-positive throughout (Fig. 2A). Double labeling with anti-TULP1 and anti-recoverin revealed that the cells reactive for recoverin (presumed photoreceptors) were restricted to the outer half of the neuroblast layer (Fig. 2B), whereas TULP1-labeling was also found in undifferentiated progenitor cells in the inner neuroblast layer (Fig. 2C). A thinner layer of cells at the outermost margin of the retina, just opposite the RPE, was intensely reactive for both TULP1 and recoverin (Fig. 2C).

In the retina at fetal week 17.4 and later, there was a prominent layer of differentiating photoreceptors in both central and peripheral retina, all of which were reactive for both TULP1 (Figs. 2D through 2G) and recoverin. Among these cells, some had large, prominent cell bodies that protruded into the subretinal space (Figs. 2D, 2E) and a basal process typical of immature cones.16 These differentiating cones were intensely positive for TULP1, and at 11 weeks and later, some were reactive for blue cone opsins (Fig. 2F). The first cones labeled with anti-red/green cone opsin were found at age 15.4 weeks, and they were also strongly positive for TULP1 (not shown). By 17.4 weeks, all cones in the centralmost retina were intensely TULP1-positive (Fig. 2G). At this time, the differentiating rods around the developing fovea formed a layer vitread to the differentiating cones, but these early rods were only weakly reactive for TULP1 (Fig. 2E).

A second group of TULP1-positive cells was found in the developing retinas at fetal age 15.4 weeks to 1 month after birth. These cells lay in the nerve fiber and ganglion cell layers and were often found in small groups, particularly in the peripheral retina (Figs. 2E, 2G, 2H). These cells were larger...
than the neighboring ganglion cells and were round or oval with numerous fine processes. The location and morphology of these cells suggested initially that they were astrocytes, and double labeling was therefore performed with anti-TULP1 and anti-GFAP. Although numerous stellate astrocytes were labeled with anti-GFAP, the TULP1-reactive cells in the innermost retina were GFAP-negative. The multipolar TULP1-reactive cells were labeled with anti-recoverin (Fig. 2I), and some were reactive with anti-blue cone opsin (Fig. 2F), but not with anti-red/green cone opsin or anti-rhodopsin. Similar recoverin-positive multipolar cells have been described in the ganglion cell and nerve fiber layers of fetal human retinas, although cone-specific markers were not used in that study.

Because of the optic disc abnormalities observed in young human RP patients with TULP1 mutations, it was anticipated that ganglion cells and the optic nerve head might show immunolabeling with anti-TULP1. Scattered cells in the ganglion cell layer were weakly TULP1-positive from 11 weeks onward (Figs. 1F, 2E, 2G); however, they were always less intensely labeled than the differentiating photoreceptors in the same area. In adult retinas, a few ganglion cells and inner nuclear layer cells were also weakly TULP1-positive (Figs. 1A, 1D). The optic nerve head and astrocytes, evaluated with anti-GFAP, were consistently negative for TULP1 (not shown).

**Postnatal Human Retinas**

The pattern of TULP1-labeling in the newborn (3 day and 1 month) retinas was essentially the same as in the adult. Strong TULP1-reactivity was found in the cone and rod inner segments, somata, and synapses, and a few inner nuclear layer and ganglion cells were weakly labeled. In the periphery, TULP1-and recoverin-positive cells were present in the nerve fiber layer, and a few of these were reactive for blue cone opsin. The optic nerve head and astrocytes were TULP1-negative.

**Discussion**

The present study documents the localization of TULP1 in developing and adult human retinas. The finding of very early expression of TULP1 by retinal neuroblasts at 8.4 fetal weeks (the earliest age examined) suggests a fundamental role for TULP1 in retinal differentiation. Further, the observation that all retinal neuroblasts were TULP1-positive indicates that this protein may be involved in development of both photoreceptors and inner retinal neurons. TULP1-labeling was strongest in developing cones, including displaced blue cones in the nerve fiber layer, whereas in adult retinas the labeling was somewhat less intense in cones than in rods. The significance of these cone/rod differences is unknown but suggests that both populations of photoreceptors are probably affected early in development of the retina in RP patients with mutations in the TULP1 gene. In agreement, recent studies of patients homozygous for a TULP1 mutation and TULP1−/− mice have documented early degenerative changes in the cones as well as the rods.

Many genes associated with human RP are expressed solely in photoreceptors, the cells that usually undergo primary degeneration. Older patients with RP often show optic disc changes consistent with secondary degeneration of ganglion cells and their axons. Sahly et al. found tub expression in ganglion cells during mouse retinal embryogenesis but mainly in photoreceptors in postnatal and adult mice. The observation that the Dominican Republic patients with RP who are homozygous for a splice-site mutation in the TULP1 gene can show optic disc degeneration in the first decade of life raised the question of whether TULP1 is expressed in inner retinal neurons as well as photoreceptors during development. Findings from the present immunolabeling study favor this supposition, because TULP1-labeling was detected in differentiating ganglion cells early in retinal development and in a few of these cells in adult retinas.
The absence of TULP1-labeling in cone and rod outer segments found here in human retinas and also in wild-type mouse retinas suggests that this protein does not play a role in phototransduction. It is critical to identify the genes regulated by TULP1 as a possible transcription factor to understand the degenerative changes in photoreceptors and inner retinal neurons in RP caused by mutations in the TULP1 gene.

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References