Fas-independent Apoptosis of Photoreceptor Cells in C₃H Mice

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Abstract: The morphogenesis of the photoreceptor cells in the retinas of C₃H mice carrying the *rd* gene and C₅₇BL mice carrying the normal gene was compared, and retinas of the C₃H mutants (C₃H-*lpr/lpr*, -*lpr^{cg}/lpr^{cg}*, and -*lpr/lpr-gld/gld*) defective in apoptosis through the Fas system were examined. In the C₅₇BL retina, the inner and outer nuclear layers were separated at 8 days of age, and the photoreceptor inner and outer segments began to grow between 8–11 days after birth with their most rapid growth occurring between 14–17 days of age. In the C₃H retina, the development was comparable to that of the C₅₇BL retina at 8 days of age but the reduction in thickness of the outer nuclear and photoreceptor layers was noted at 11 days of age, and the outer nuclear layer became reduced to only a few nuclei in thickness at 14 days, being completely missing or reduced to a single row of cells at 20 days. The degeneration was by an apoptotic mechanism as confirmed morphologically and by the TUNEL method. In all the C₃H mutant retinas examined over 24 days of age, the complete depletion of the outer nuclear layer or reduction to a single row comparable to 20-day-old C₃H mice was seen. The *rd* gene action is therefore independent of Fas/Fas ligand-mediated apoptosis.

Key words: Apoptosis, C₃H, Fas, retinal degeneration, *rd* gene

Introduction

Keeler (1924) first described the hereditary lack of the photoreceptor cells in mice derived from the Bagg albino strain, and established that it is inherited as a recessive Mendelian character [9]. This character was subsequently found in various mouse colonies, but strains carrying the Keeler abnormality seem to have been lost in the following decades with the exception

of BDB and P/JAX strains [16]. Brückner (1951) later reported a similar hereditary retinal abnormality in albino mice caught near Basel [2]. Dunn (1954) incidentally found the same character in C₃H mice [6]. The C₃H mouse is well pigmented and the eyes lack any distinct abnormality. They are alert and move around so skillfully that the lack of vision is not apparent. Keeler described the abnormality as “rodless retina”, and since the 1950s, several other investigators

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referred to it as "retinal degeneration" and designated "*rd*" as a gene symbol [10]. C₃H has the *rd* allelic gene [14]. The retina in the *rd* mice starts developing normally and is indistinguishable from that in normal mice until the onset of photoreceptor outer segment development, when the photoreceptor cells degenerate [12]. Apoptosis is involved in cell death during normal retinal development [28], and is also involved in photoreceptor cell death in *rd* mice [4, 13, 17, 18]. Dying cells can be identified morphologically and can be localized by the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method.

Various mechanisms and intracellular signaling pathways are involved in apoptosis. Among them, the Fas antigen is one of the most well characterized cell surface proteins that mediate apoptotic signals into the cell [26]. The *lpr* (lymphoproliferation) and *lpr^{cg}* (*lpr* complementing *gld*) mutations on chromosome 19 [25] are a defective allele of the Fas locus characterized by no expression of the Fas antigen and expression of the point-mutated nonfunctional Fas antigen, respectively [11, 26]. The *gld* (generalized lymphoproliferative disease) locus on chromosome 1 encodes a ligand for the Fas antigen and the *gld* is a point-mutation of the locus leading to the expression of nonfunctional ligand [19, 23]. The absence and non-functioning of the Fas antigen in *lpr/lpr* and *lpr^{cg}/lpr^{cg}* mice, respectively, and the non-functioning of the ligand in *gld/gld* mice arrest apoptosis via the Fas pathway. The Fas antigen is expressed in thymus, heart, liver and ovaries [27]. The relationship between Fas and retinal degeneration on the C₃H background has not yet been studied. We have therefore comparatively studied the postnatal retinal development of C₃H and C₅₇BL mice, and examined the retinas of *lpr*- and *lpr^{cg}*- homozygous and *lpr-gld*-double homozygous mutants on the C₃H background. To compare the *rd* phenotype, colored C₅₇BL strain mice, which carry the normal allele or an otherwise normal retina, served as the control.

Materials and Methods

Animals: C₅₇BL/6N and C₃H/HeJ (C₃H-+/+) purchased from Clea Japan were bred and maintained in the Animal Research Center, Kansai Medical University. C₃H/HeJ-*lpr/lpr* (*lpr*) and C₃H/HeJ- *gld/gld* (*gld*) mice were purchased from the Jackson Laboratory (Bar

Harbor, ME) and have been maintained in the Laboratory Animal Research Center, Institute of Medical Science, the University of Tokyo. CBA/KJms-*lpr^{cg}/lpr^{cg}* mutants were originally found by Matsuzawa *et al.* [15]. C₃H/HeJ-*lpr^{cg}/lpr^{cg}* (*lpr^{cg}*) mice were established by introducing the *lpr^{cg}* gene from a mutant mouse into C₃H/HeJ by more than 10 generations backcrossed by Matsuzawa. C₃H/HeJ-*gld/gld-lpr/lpr* (*gld-lpr*) double mutant mice were obtained by interbreeding (*lpr* × *gld*) × *gld* mice with lymphadenopathy and selecting the mice with no Fas expression from the progeny by flow cytometry. All animals were maintained at 22 ± 2°C in a 12-hr light/12-hr dark environment with unlimited access to commercial pellet and water. The illumination level was under 60 lux inside the cage. Each progeny was used for the observation.

Experimental design: C₅₇BL and C₃H mice were killed by cervical dislocation at 8, 11, 14, 17 and 20 days of age. Eight eyes from 4 individuals were examined at each age point. C₃H mutants (*lpr*, 3 mice; *lpr^{cg}*, 4 mice; *lpr-gld*, 4 mice) were randomly killed at from 24 to 155 days of age. Both males and females were included in the study.

Tissue Processing: The eyes were immediately removed and immersed in neutral buffered formalin, methacarn or 1/2 Karnovsky solution. For light microscopy, formalin- and methacarn-fixed tissues were embedded in paraffin and stained with H & E. Formalin-fixed tissues were applied for TUNEL staining by using an *in situ* apoptosis detection kit (Apop-Tag, Oncor, Gaithersburg, MD, USA) according to the manufacturer's instruction. For electron microscopy, tissues fixed in 1/2 Karnovsky fixative were post-fixed in OsO₄, and embedded in Luvac 812. All sections were cut perpendicular to the plane of the pigment epithelium.

Quantitative analysis: The thickness of the different layers in the retina was measured sequentially near the posterior pole of the retina, and the % TUNEL-positive cells were determined for the whole retina.

Results

Morphogenesis of the retina in C₅₇BL mice and C₃H mice: Development of the C₃H retina was comparable to that of the C₅₇BL retina at 8 days after birth (Figs. 1a and f). At this stage, the retina of both strains was

histologically indistinguishable in the size and degree of maturation of the cells and the thickness of each layer. The inner and outer nuclear layers were completely separated, but the photoreceptor layer was still thin. In 11-day-old C₅₇BL mice, the retina increased in thickness due to continuing growth of the inner plexiform layers and the photoreceptor layer (Fig. 1b). The nuclei of both inner and outer nuclear layers appeared well differentiated by this age. At 11 days, a difference in development was first detected in the C₃H mouse (Fig. 1g). The growth in the thickness of the retina was clearly less pronounced, largely due to the depletion of the outer nuclear cell layer, and the photoreceptor layer did not develop. The photoreceptor nuclei of the C₃H retina were more densely stained and their chromatin more clumped. Although the outer nuclear layer degenerated, the other layers continued to differ-

entiate as in the C₅₇BL mouse. From 11 days on, in the C₅₇BL mouse, the inner and the outer nuclear layers did not show any further increase in thickness; on the contrary, the thickness of these layers slowly decreased. The thickness of the outer nuclear layer and photoreceptor layer was significantly different in the C₃H and C₅₇BL mice at day 14 (Figs. 1c and h). The outer nuclear layer had undergone extensive degeneration and decreased in thickness. In the C₅₇BL retina the outer nuclear layer was about 10-nuclei thick, but in the C₃H retina it was only 3- to 4-nuclei thick. The outer nuclear layer was therefore reduced to less than half of its original thickness in C₃H mice. The photoreceptor segments appears to show the same degree of depletion. From this age on, compared with the C₅₇BL, the reduction of the outer nuclear layer in the C₃H retina continued progressively, the outer nuclear layer was reduced to about

Fig. 1. Changes in the retina in C₅₇BL and C₃H mice at 8 to 21 days of age. H & E, $\times 200$.

1 to 2 rows of nuclei at 17 days (Figs. 1d and i), and finally, at 20 days of age, it was reduced to a single layer of degenerating nuclei or was completely missing (Figs. 1e and j). The layer of pigment epithelium was a single row of cells already developed at 8 days after birth and did not show any appreciable change during the observation period. Fig. 2 illustrates the relative changes in the various retinal layers during development of the normal *C₅₇BL* mouse, and the *C₃H* mouse carrying *rd* mutation. Postnatal development of the *C₃H* retina involved the reduction in the thickness of the outer nuclear layers as well as photoreceptor layers without involvement of the other layers. The photoreceptor cells selectively degenerated leaving a single

remnant or were completely lost at 20 days of age. During the course of photoreceptor degeneration, no evidence of inflammation was observed within the retina.

The number of TUNEL-positive cells in the outer nuclear layer of the *C₅₇BL* and *C₃H* retina was compared at 8 to 20 days of age (Fig. 3). The percent of positive cells was low in the *C₅₇BL* retina, whereas many nuclei in the outer nuclear layer were stained in the *C₃H* retina, reaching a maximum at 14 days but decreased at 20 days of age. The morphology of the *C₃H* retina was evaluated by electron microscopy at each stage. Beginning from 11 days after birth, degenerating cells with pyknotic nuclei were dispersed between cells possessing a nuclear pattern of euchro-

Fig. 2. Sequential changes (%) in thickness of the different layers in the posterior pole of the retina in *C₅₇BL* and *C₃H* mice. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; PEL, pigment epithelial layer.

matin characteristic of normal photoreceptors (Fig. 4). The cytoplasm of degenerating cells was shrunken, making visualization of their cytoplasmic content difficult. These degenerating cells had increased in number at 14 days of age. At 17 days, few rows of photoreceptor cells, some of which were apoptotic, existed between the inner nuclear layer cells and the pigment epithelium (Fig. 5). Finally, at 20 days of age, the inner nuclear cells were in direct contact with the pigment epithe-

lium in some areas.

Retina in C₃H mutant mice: In all the *lpr*, *lpr^{cs}* and *lpr-gld* mice, the retina was affected similarly to that in the C₃H-+/+ mice. From 24 days of age on, the outer nuclear layer was a single row outer nuclear layer, or was completely missing. Again, the defect in the retinas of these animals appeared to involve only the photoreceptor elements (Fig. 6). In spite of the absence of photoreceptor cells, the remaining retinal layers seemed to develop normally. In the mutant mice, the reduction in the thickness of the outer nuclear layer

Fig. 3. Sequential comparison of % TUNEL-positive cells in the outer nuclear layer of C₅₇BL and C₃H mice at 8 to 20 days of age.

Fig. 4. Apoptotic photoreceptor nuclei within the outer nuclear layer of an 11-day-old C₃H mouse. Note the degenerating cells with pyknotic nuclei. $\times 5,000$.

Fig. 5. Electron micrograph of a 17-day-old C₃H mouse. There are apoptotic photoreceptor cells between the inner nuclear cells and pigment epithelium. $\times 2,500$.

Fig. 6. C₃H-*lpr/lpr* retina at 24 days of age. Note the depletion of photoreceptor cells. H & E, $\times 200$.

was more evident in the posterior pole of the retina than in the peripheral retina.

Discussion

Retinitis pigmentosa is included in a group of inherited human diseases in which photoreceptor degeneration leads eventually to blindness [8]. Animal models for human retinitis pigmentosa have been documented [3]. Among them, albino mice of the 020/A strain carry an autosomal recessive retinal degeneration slow (*rds*) gene located on chromosome 17 [5, 24]. Mice homozygous for the *rds* gene fail to develop photoreceptor outer segments and show signs of slow degeneration of the retina and complete lack of photoreceptor cells at approximately 12 months of age [21]. In heterozygous *rds*, partial loss of photoreceptor cells starts later than in the homozygous *rds* and progresses at a much slower rate [7]. By contrast, photoreceptor degeneration occurs very early and progresses extremely rapidly in C₃H mice carrying the autosomal recessive *rd* gene on chromosome 5 [22]. The early stages of morphogenesis of the photoreceptor cells start in the usual way, but the degeneration of photoreceptor cells results in a final depletion state at 20 days of age. Visual alteration in *rd* mice is not the result of a primary arrested photoreceptor cell development, but of a secondary change in the differentiating photoreceptor cells.

The general process of the retinal degeneration in the C₃H mice described earlier [6, 12, 16, 20] was confirmed in the present study. The specific spatial pattern of photoreceptor cell death occurs through an apoptotic mechanism with a central to peripheral gradient. In agreement with previous reports [4, 13, 17, 18], a high percentage of TUNEL-positive nuclei was seen in the C₃H photoreceptor cells. Morphologically, nuclear condensation and pyknosis occurred but the morphologic features did not entirely fit the criteria defined by Kerr and coworkers such as nuclear chromatin condensation at the periphery [1]. Distinct morphological difference may exist among cells undergoing apoptosis.

In *lpr*, *lpr^{cg}* and *gld* mice, apoptosis via the Fas pathway is arrested. Retinal degeneration was not inhibited at all in any of the Fas/Fas ligand-deficient mice, demonstrating that Fas and Fas ligand do not play a primary role in the *rd* disease. It is important to determine what other apoptotic signaling pathways are involved in pho-

photoreceptor cell death in C₃H mice. This will contribute to better understanding of the genesis of retinitis pigmentosa in humans.

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