A PATHOLOGIC STUDY OF DEGENERATION OF THE ROD AND CONE POPULATIONS OF THE RHODOPSIN PRO347LEU TRANSGENIC PIGS*

BY *M.O.M. Tso*, MD, DSc, *W.W.Y. Li*, MD (BY INVITATION), *C. Zhang*, MD (BY INVITATION), *T.T. Lam*, PhD (BY INVITATION), *Y. Hao* (BY INVITATION), *R.M. Petters*, PhD (BY INVITATION), AND *F. Wong*, PhD (BY INVITATION)

ABSTRACT

Purpose: Transgenic pigs with rhodopsin (Pro347Leu) mutation exhibited rod-cone degeneration. We compared the pathologic characteristics of the rod degeneration versus those of the cone cells.

Methods: The posterior and peripheral retinas of these transgenic pigs of age 4, 6, 8, 12, 24 and 33 weeks and normal pigs of age 4 and 8 weeks were studied by light and EM and morphometry.

Results: The pathologic changes observed in the posterior and peripheral retinas of the transgenic pigs could be conveniently described in 3 phases: I) an initial phase of rapid and extensive degeneration of the rod cells in the first 6 weeks of age; II) an acute phase of cone cell degeneration involving approximately half of the population and lingering rod degeneration in the 6 to 12 weeks of age; and III) a partial cone recovery to be followed by a chronic degenerative phase of the remaining cones cells from 12 to 33 weeks of age.

Conclusion: Our study showed that the degenerative changes of rod cells could be differentiated from those of the cone cells. Cone and rod populations degenerated along different time schedule with different pathologic features. Hence, treatment for retinitis pigmentosa might vary with the different stages of the disease.

*From the Chinese University of Hong Kong (Drs Tso, Li, Zhang, and Lam), Duke University (Ms Hao and Dr Wong), and North Carolina State University (Dr Petters). Supported by the Research Grant Council of Hong Kong, Fight for Sight Foundation of Chinese University of Hong Kong, NIH grants EY09738 and EY09047, Foundation Fighting Blindness of Baltimore, RBP Incorporated of New York and a gift from Santen of Japan.

INTRODUCTION

Even though over 100 mutations of the rhodopsin, peripherin, and other genes have been identified in patients suffering from retinitis pigmentosa (RP),[1-4] the cellular mechanisms by which these mutations cause degeneration of the photoreceptor cells have not been determined. Even more puzzling is the fact that many genetic mutations of rod proteins eventually lead to cone cell death. The pathologic processes and the mechanisms of cone degeneration are not known. In RP patients, the loss of rod cells initiates at the periphery of the retina, but eventually both rod and cone cells from both the peripheral and macular regions of the eye degenerate. The relationships between the degenerative processes of these 2 groups of cells in different geographic locations need to be further examined.

The most incapacitating insult to the retina of an RP patient is the loss of central vision because of degeneration of the cone cells in the macular area. Any therapeutic approach to delay the death of the cone cells would be immensely beneficial to the quality of life of these patients. The key to explore new therapeutic approaches to this disease is to develop an animal model of retinitis pigmentosa, which would allow a detailed study of pathogenetic mechanisms or pathologic process of rod-cone degeneration. The many naturally occurring or genetically engineered rodent models of retinitis pigmentosa, which include rodents with rhodopsin or peripherin mutations and rhodopsin knockout mice,[5-7] have few cone cells. The absence of macula or area centralis in these rodent models further hampers exploratory research of cone-cell degeneration. Recently, Petters, Wong and associates[8] developed a transgenic pig model that expresses rhodopsin (PRO347LEU) mutation. Since the pig has a humanlike cone-rod retina,[9,10] we explored the pathologic processes involving these 2 cell populations in these transgenic pigs.

METHODS AND MATERIALS

To create the rhodopsin Pro347Leu transgenic pig model, a DNA construct containing all the coding regions of the porcine rhodopsin gene and its flanking sequences was microinjected into pig embryos. Details about the production and initial characterization of these pigs were reported elsewhere.[8] Two lines corresponding to 2 different integration sites were established: Pig-Tg N1 Pet and Pig-Tg N2 Pet. The phenotypes of these two lines were similar. Seven Pig-Tg N1 Pet pigs were studied by light and electron microscopy in this study. The posterior and peripheral retinas of these transgenic pigs, aged 4, 6, 8, 12, 16, 24, and 33 weeks, were examined. In addition, the retinas of 2 normal pigs, aged 4 and 8 weeks, served as control. After euthanasia, the pigs were enucleated and the retinas were

fixed in 0.5% glutaraldehyde and 4% glutaraldehyde in 0.13M phosphate buffer, pH 7.4 at room temperature, osmicated, dehydrated by ascending concentrations of alcohol, and embedded in epoxy resin. Sections for light microscopy were stained with 1% toluidine blue, and ultra-thin sections for electron microscopic study were treated with uranyl nitrate and lead citrate.

RESULTS

The pathologic changes observed in the posterior and peripheral retinas of the transgenic pigs (Fig 1) could be conveniently described in 3 phases: (1)

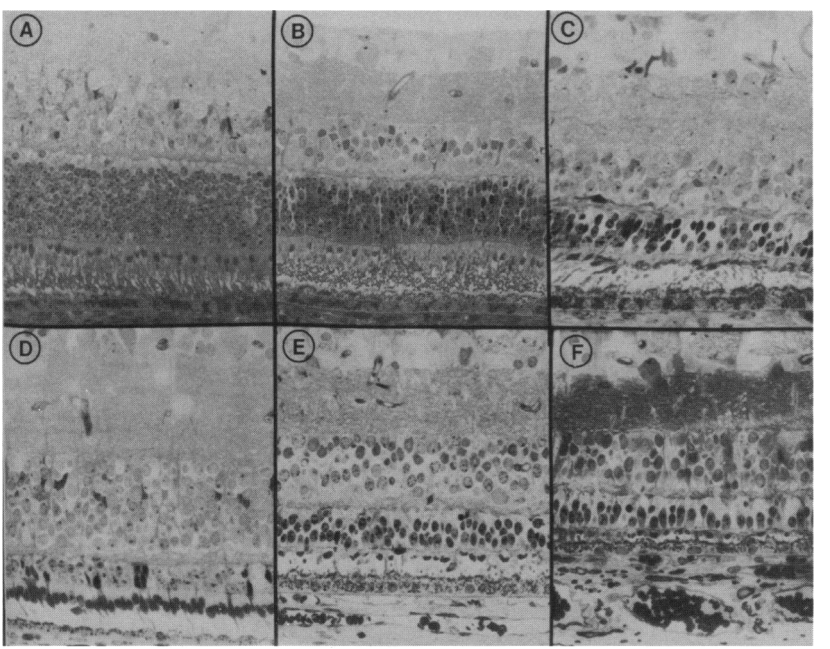

FIGURE 1

Peripheral retina of normal pig at 4 weeks of age (A) and peripheral retina of transgenic pigs at 4, 6, 8, 12, and 24 weeks of age (B-F). A, Retina of a 4-week-old normal pig. B, At 4 weeks of age, outer nuclear layer is thin with scattered densified nuclei. C, At 6 weeks of age, outer nuclear layer is markedly thin, reducing to 2 to 3 nuclear thick. Many densified nuclei are surrounded by watery cell processes of Müller cells. Inner segments are shortened, and outer segments are sparse. Retinal pigment epithelium remains unremarkable. D, At 8 weeks of age, outer nuclear layer of retina is reduced to 2 nuclear thick, and many of the densified nuclei have disappeared. However, scattered cone nuclei, about outer limiting membrane, appear densified, and so are cone inner segments and cone pedicals. E, At 12 weeks of age, most of densified nuclei of both rod and cone cells have disappeared. Short outer segments are seen. F, At 24 weeks of age, most of cone cells stretched from external limiting membrane to middle limiting membrane with markedly shortened inner segments and outer segments. Pigment epithelium remains unremarkable.

an initial phase of acute and extensive degeneration of the rod cells in the first 6 weeks of age, (2) an acute phase of cone cell degeneration involving approximately half the cone population associated with lingering rod degeneration at 6 to 12 weeks of age, and (3) a chronic degenerative phase of the remaining cone cells from 12 to 33 weeks of age. In general, the degeneration of the rod and cone cells in the peripheral retina was more severe when compared with that in the posterior pole (Figs 1 and 2), and the cone cells appeared better preserved in the posterior pole. The 3 phases also overlapped with each other.

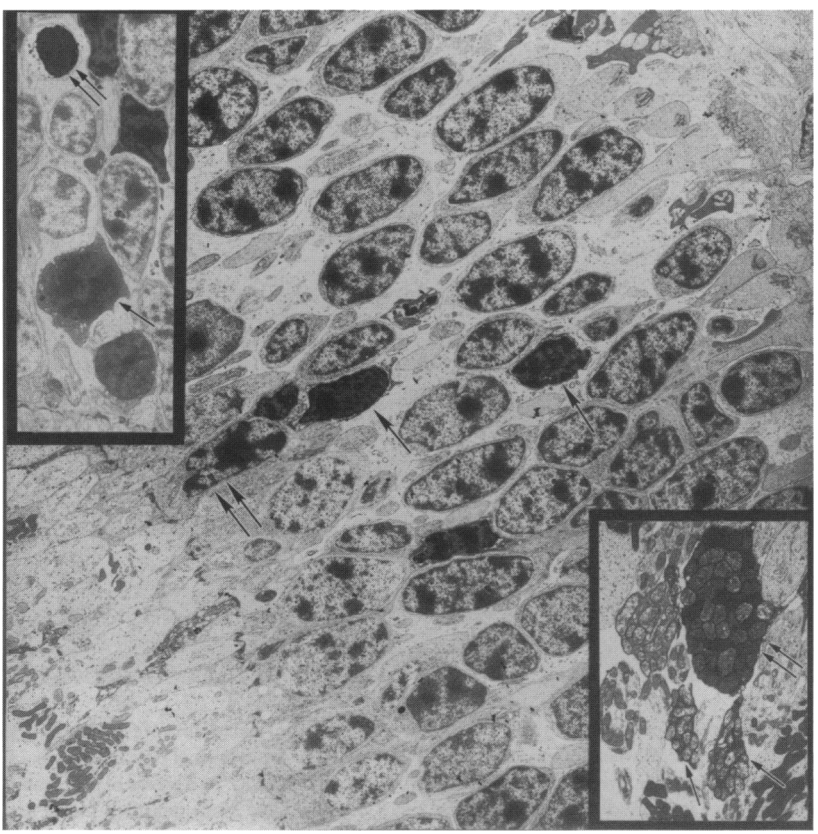

FIGURE 2

Outer nuclear layer of transgenic pig at 4 weeks of age. Noteworthy are scattered densified rod nuclei (single arrow) with clumped chromatin and small amount of densified cytoplasm and synaptic terminals. One cone nucleus (double arrows) shows mild clumping of chromatin material. Left upper inset shows uniformly densified cytoplasm around nuclei with clumped chromatin (single arrow) and one densified nucleus (double arrows) with edematous and lysed cytoplasmic contents. Right lower inset shows degenerating cone (double arrows) and rod (single arrows) inner segments. Cytoplasm of these degenerating cells is densified even though mitochondria appear relatively unremarkable.

At 4 weeks of age, the outer nuclear layer of the retina of the transgenic pigs was thinner than that of the normal controls (Fig 2). Scattered rod cells exhibited shrunkened nuclei with clumped chromatin, and densified cytoplasm in the perikaryon, inner segments, and synaptic terminals. Few cone cells were affected. The inner and outer segments appeared mildly sparse, while the retinal pigment epithelium was unremarkable.

At 6 to 8 weeks (Fig 3), the degeneration of the photoreceptor cells accelerated involving predominantly rod cells with some cone-cell involvement. Not only was the outer nuclear layer thin, but many of the missing

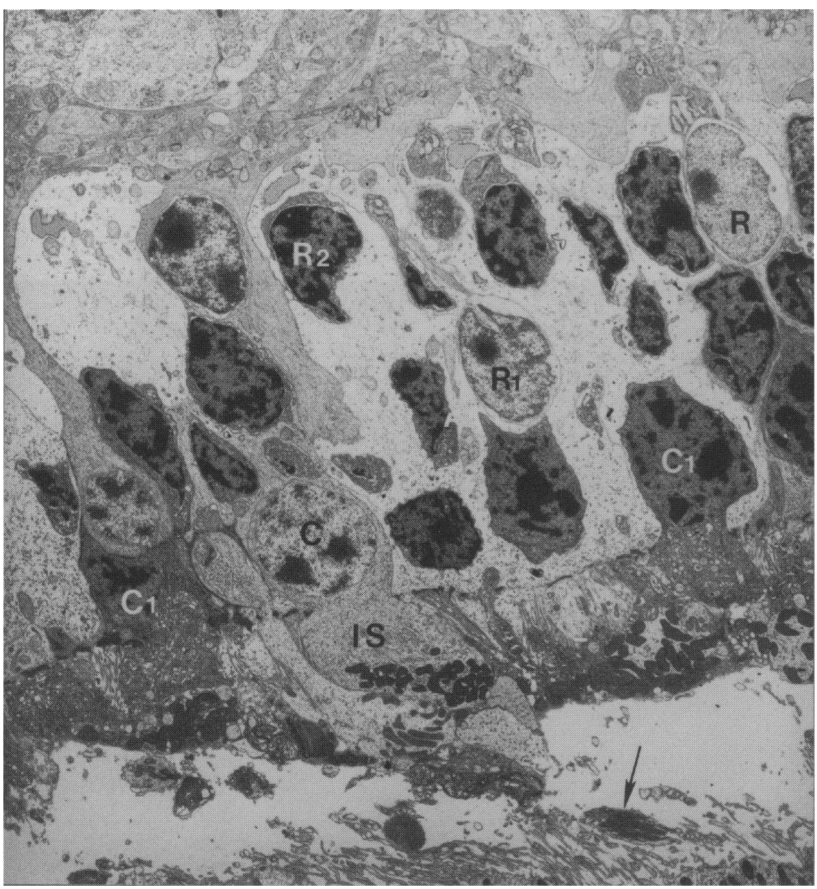

FIGURE 3

Outer nuclear layer of transgenic pig at 6 weeks of age. Noteworthy is marked loss of photoreceptor nuclei with abundant edematous Müller fibres. Nuclei exhibit various stages of degeneration. Inner segments are much shortened. Two of the cone cells (C_1) show densified cytoplasm with mildly dilated smooth endoplasmic reticulum. In contrast, relative intact cone cell (C) can be seen with shortened inner and outer segments. Outer segments (arrow) are markedly shortened and disorganized.

nuclei were replaced by cell processes of Müller cells, which exhibited watery cytoplasm. The rod nuclei showed varying stages of degeneration, ranging from infolding of the nuclear membrane and peripheral clumping of the chromatin material, to shrunken nuclei and disrupted nuclear membrane and karyorrhexis (Fig 4). The cone nuclei also had clumped chromatin, intensely densified cytoplasm, and dilated endoplasmic reticulum, but the mitochondria appeared intact (Fig 3). The inner segments became short, the outer segments were sparse, and the photoreceptor elements were irregularly aligned. At 8 weeks of age (Fig 5), most of the rod nuclei had disappeared. A few degenerating cone cells could still be seen with lengthened and realigned inner and outer segments. The degenerating cone cells exhibited shrunken nuclei and dense and clumped chromatin material. The latter was seen to be scattered in the perikaryon as the nuclear membrane ruptured. The cytoplasm of the perikaryon, cone pedicals, and inner segments of the degenerating cells was characteristically densified with dilated endoplasmic reticulum. The retinal pigment epithelium was unremarkable.

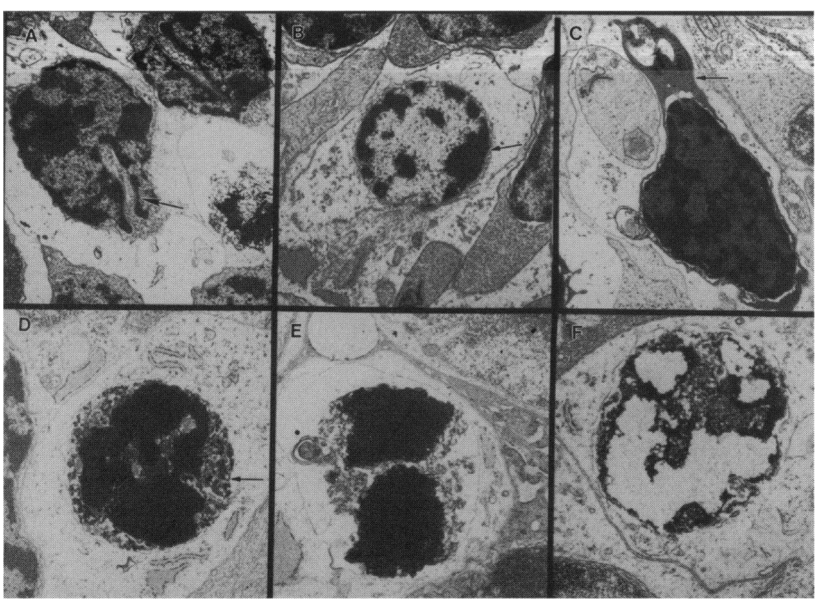

FIGURE 4

Outer nuclear layer of transgenic pig at 6 weeks of age. A, Marked infolding of nuclear membrane of photoreceptor cells. B, Peripheral clumping of chromatin material. C, Shrunken nuclei with densified cytoplasm of a rod cell. D, Disrupted nuclear membrane with granular cytoplasm of a rod cell. E, Disintegration of chromatin material of a degenerating rod nucleus with granular cytoplasm is separated into two masses with lysis of plasmalemma. F, Final stage of degeneration of rod nucleus showing loss of chromatin material.

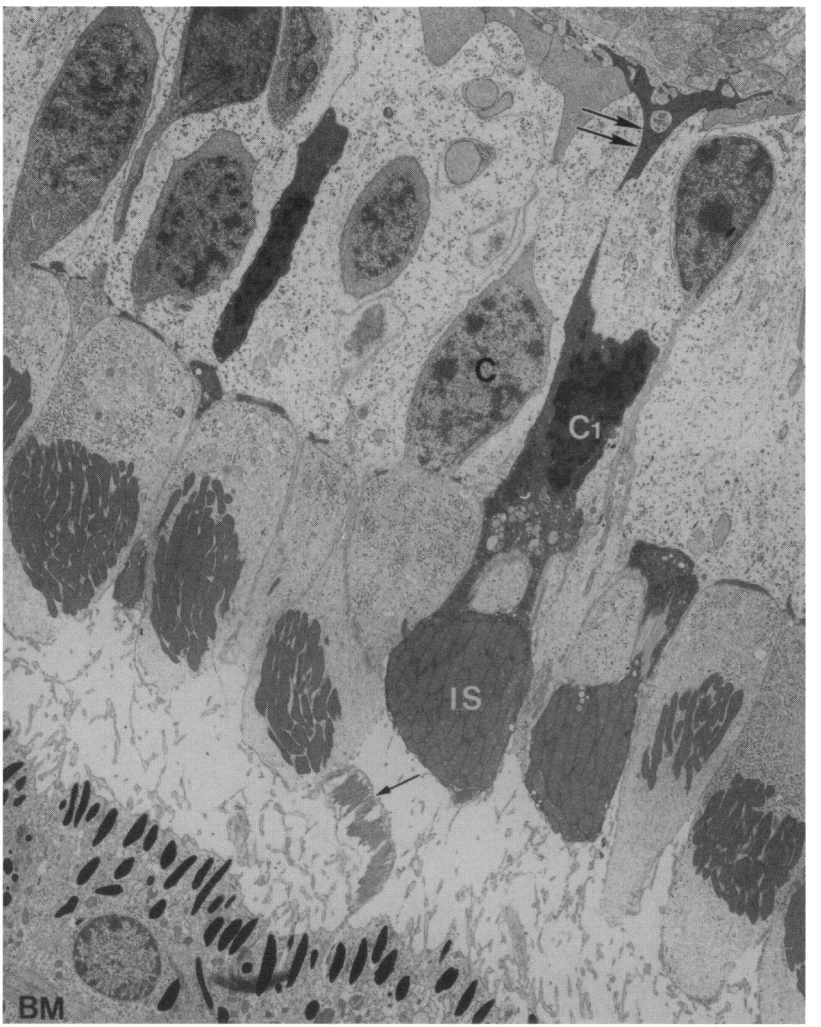

FIGURE 5

Outer nuclear layer of retina of transgenic pig at 8 weeks at age. Noteworthy is extensive loss of rod nuclei. Cytoplasmic processes of Müller cells have greatly expanded to fill intercellular space. A few degenerating cone cells (C_1) comparing with a normal cone cell (C) exhibit shrunken nuclei with clumped chromatin material and extensively densified cytoplasm in perikaryon, cone pedical, and inner segment. Mitochondria exhibit intact membrane. Inner segments of remaining cone cells are aligned with short outer segments. Retinal pigment epithelium is unremarkable.

At 12 to 24 weeks, most of the rod cells had disappeared, and the cone cells stretched across the entire outer nuclear layer (Fig 6). The inner and outer segments of the remaining cone cells were realigned but continued to be more shortened and fragmented in the peripheral than the central retinas (Figs 7 and 8). Mild proliferative reaction of the retinal pigment epithelium was first noted at 24 weeks of age. At 33 weeks of age, the remaining cone cells in peripheral and posterior retina were unchanged from those at 24 weeks of age.

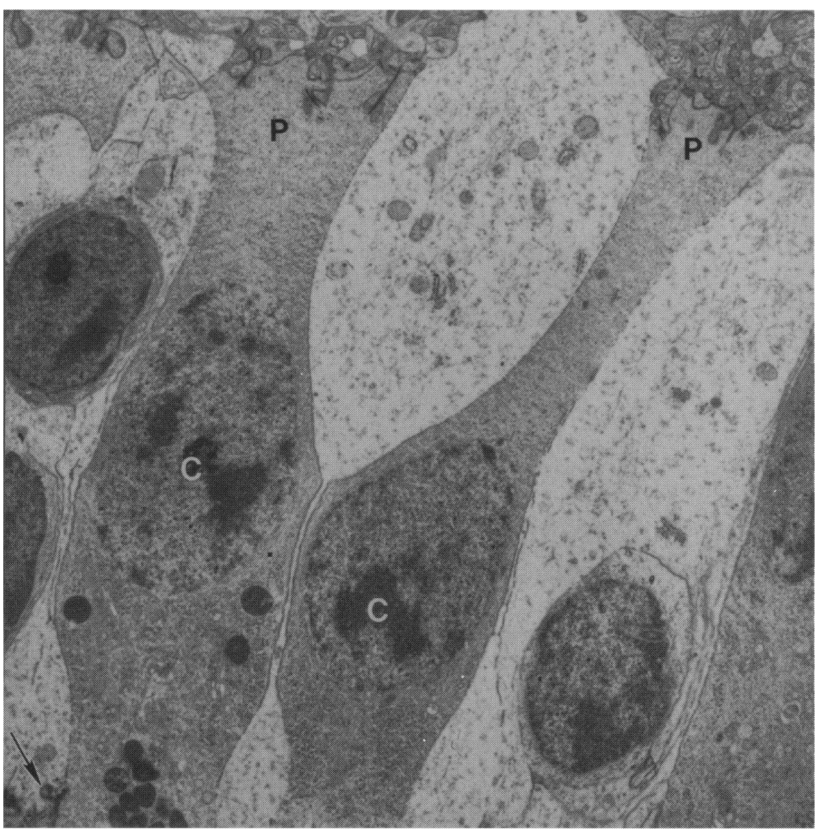

FIGURE 6
Outer nuclear layer of retina of transgenic pig at 24 weeks of age. Degenerating rods nuclei are absent. Remaining plump cone cells (C) stretch across entire outer nuclear layer. Cones pedicals (P) appear intact. External limiting membrane is indicated by arrow.

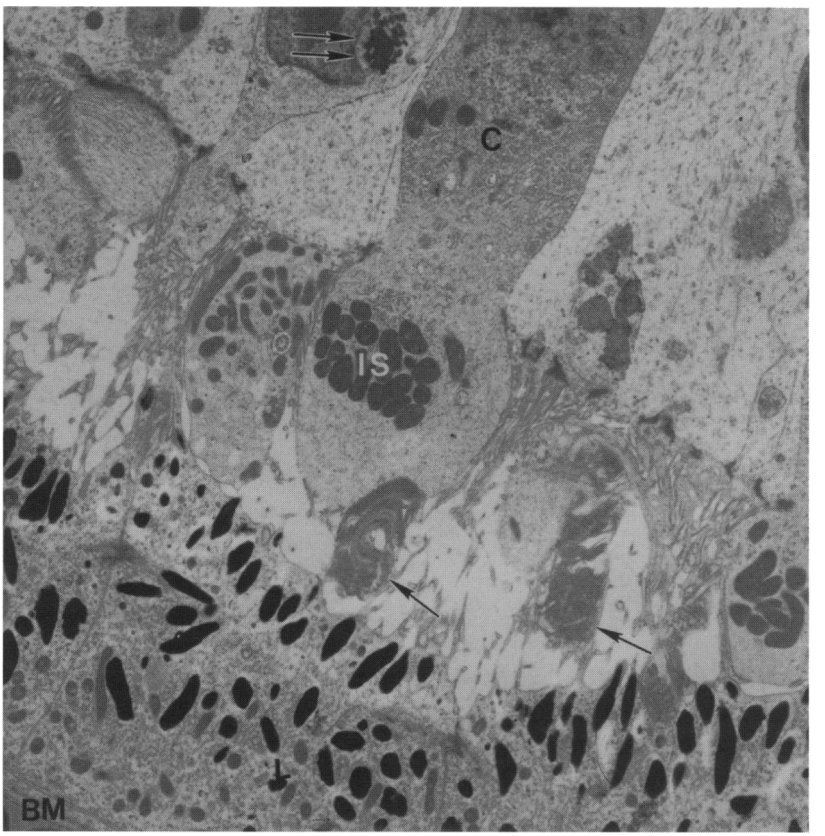

FIGURE 7
Inner and outer segments of these cone cells of peripheral retina are short and stubby. Outer segments are irregularly aligned. Noteworthy is cellular debris (double arrows) phagocytosed within cytoplasm of cone cell. BM, Bruch's membrane.

DISCUSSION

Pathologic study of human retina with retinitis pigmentosa has been frustrating,[11-16] not only because of the wide spectrum of the disease processes within this umbrella entity, but also because of the large individual variations in severity and rate of progression as well as geographic and genetic differences. In addition, most of the human retinas for pathologic study were available only at the late stage of the disease, when the acute degenerative changes were over and only a gliotic retina remained. The transgenic pigs with a cone-rod retina provided an unusual opportunity to examine the entire pathologic processes in different phases in the development of the disease.

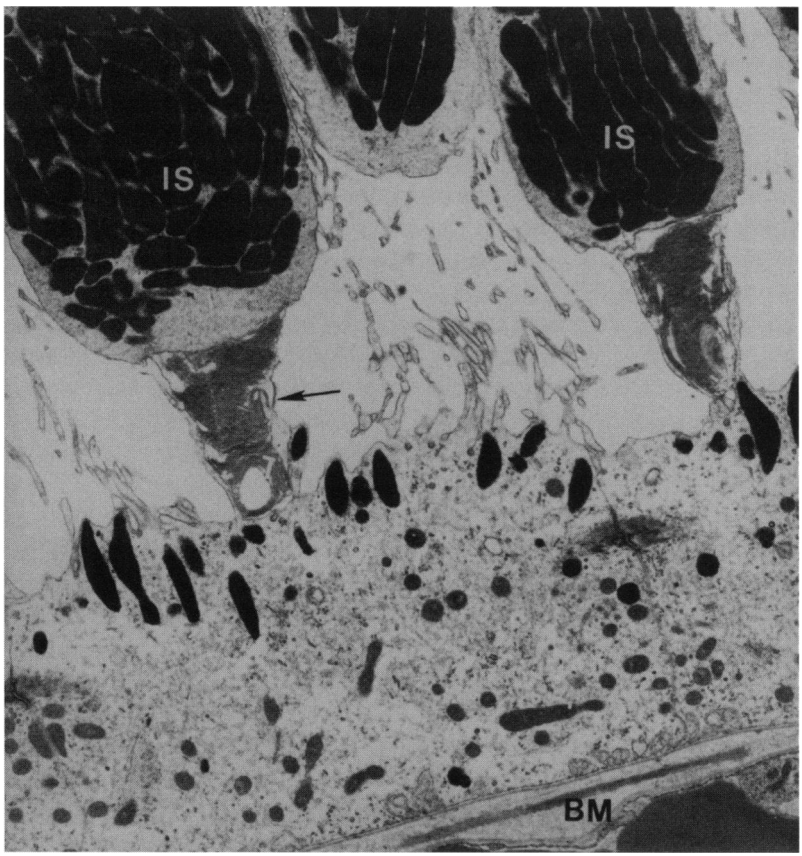

FIGURE 8
Photoreceptor elements of cone cells in posterior pole show realignment and longer outer segments when compared with those of peripheral retina. BM, Bruch's membrane.

The pathologic changes of the retina of the transgenic pigs could be conveniently described in 3 phases. In phase 1, rapid and extensive degeneration of the rod cells took place in the first 6 weeks of age. At this early stage, the retinal architecture was well preserved and allowed an opportunity to observe the primary photoreceptor changes. By 6 weeks, other secondary mechanisms appeared to set in, resulting in extensive loss of rods and cones cells. Phase 2, occurring between 6 and 12 weeks of age, consisted of acute cone degeneration involving approximately half of the population. Early in this phase, degeneration of the cone cells coincided with the extensive lingering death of the rod cells, but later as the degenerative rod cells were removed, degeneration of the cone cells continued. Phase 3

was a chronic degenerative phase of the remaining cones from 12 to 33 weeks; there was recovery of the cone elements, which were realigned even though they were shortened. The degeneration of the remaining cone cells progressed slowly. In histopathologic studies of human retinas with retinitis pigmentosa, most of the case reports described pathologic features of the third phase.

In Phase 1 of this disease at 4 weeks of age, the degenerative changes centered on the nuclei of the photoreceptor cells, which exhibited clumping of the chromatin material, and densification of the scanty cytoplasm of the perikaryon, the synaptic terminals, and inner segments. We intrepreted that these changes of the rod cells were the primary consequence of the rhodopsin mutation. The mechanism of cell loss was most likely to be apoptosis, as reported in other rodent models of retinitis pigmentosa.[17-19]

At 6 weeks, the degenerative process was greatly accentuated. Many of the rod photoreceptor nuclei developed infolding of the nuclear membrane and clumping of their chromatin material. Some nuclei were shrunken, to be followed by disruption of the nuclear membrane, granular degeneration of the cytoplasm, and fragmentation of the nuclear material into separated masses. These pathologic changes were comparable to those seen in photoreceptor cells after photic injury.[20,21] In the latter situation, the photoreceptors underwent both apoptosis and necrosis. It should be noted that even though apoptosis and necrosis are characterized by different biochemical pathways, depending on the severity of the inflicting insult, apoptosis or necrosis may occur as two ends of a spectrum of pathologic changes. These photoreceptor cells of the pigs might have developed along similar degenerative processes.

At phase 2, between 6 and 8 weeks, the cone cells showed clumping of the chromatin material and intense densification of the cytoplasm of the perikaryon, inner segments, and cone pedicals. Against a background of densified cytoplasm, the dilated endoplasmic recticulum was prominent. The mitochondrial membrane at times seemed to be preserved. By 8 weeks, most of the cellular debris of the rod cell were removed. These degenerated cone cells persisted, and the nuclear membrane of some cone cells ruptured with the nuclear material scattered in the densified cytoplasm. The outer segments of the surviving cone cells were short and yet well-aligned. These ultrastructural characteristics closely resembled a degenerating cone cell of a 22-year-old man with x-linked recessive retinitis pigmentosa.[11] The similarity of the porcine degeneration of cone cells to human retinitis pigmentosa further affirmed the potential of this animal model for pathogenetic and therapeutic studies of retinitis pigmentosa. Furthermore, the ultrastructural changes of these cone cells bear resemblance to neurons undergoing "delayed cell death" secondary to excitotoxicity.[22,23]

By 12 to 33 weeks of age, most of the rod cell debris were removed. The remaining cone cells showed remarkable recovery, and the photoreceptor elements were realigned along the external limiting membrane. Short but definitive outer segments were re-formed in the subretinal space, especially in the posterior pole. At the periphery, the outer segments were short and fragmented, and proliferative reaction of the retinal pigment epithelium toward the external limiting membrane was observed. Meanwhile, the inner segments of the cone cells in the periphery were progressively shortened, and the external limiting membrane abutted the retinal pigment epithelium. Indeed, after severe photic insult, the surviving photoreceptor cells in monkey exhibited similar shortened inner and outer segments. In the previous human studies of retinitis pigmentosa, most investigators observed these pathologic changes in the retina and so noted as the characteristics of the degenerative process of retinitis pigmentosa.[11-16] From our study, it is apparent that this is only the terminal phase of the degenerative processes.

Many mechanisms of photoreceptor cell death in retinitis pigmentosa have been proposed. However, our morphologic study suggested that three mechanisms might have been involved at different stages of the disease. At the early phase, the rod cells underwent apoptosis and necrosis, which were intrepreted as two ends of the same spectrum of the disease mechanism, probably secondary to the mutant rhodopsin in the rod cells. Subsequently, the early death of the photoreceptor cells led to excessive release of neurotransmitters, causing excitotoxic effects to the neighboring rod and cone cells. This hypothesis explained that when compared with the posterior pole, there was more severe photoreceptor loss in the periphery where rod cells were more numerous and underwent massive cell death. As the rod cell debris was cleared and the excess excitatory amino acids were removed, the surviving cone cells showed moderate recovery and re-alignment. In the third degenerative phase, the slow degeneration of the cone cells exhibited morphologic features different from those of the first two types of photoreceptor death, and the pathogenetic mechanism is still unclear.

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DISCUSSION

NARSING RAO, MD. I would like to congratulate Dr Tso and colleagues on an excellent morphologic study of retinal degeneration in mutant rhodopsin (Pro347Leu) transgenic pigs, an animal model for retinitis pigmentosa. In this ultrastructural study, the authors compared the degenerative changes of the rods with the alterations observed in the cone cells. They clearly documented profound morphologic changes in the rod cells

characteristic of apoptotic cell death and the marked loss of these cells in the first 8 weeks of life. The cone cells also revealed degenerative changes, but the onset and progression of cone cell loss were delayed. It is interesting that retinal pigment epithelium remained unremarkable during the early phase of the degenerative process, even in the presence of profound changes in the photoreceptor cells.

Rhodopsin, the photoreceptor protein of the rod cell, is a 348–amino acid integral membrane glycoprotein. Biochemical and mutagenic studies have established 3 important rhodopsin domains: the cytoplasmic loops, which are important for binding and activating transducin, a protein critical to initiation of the visual cascade; the transmembrane region, important for covalent binding of 11-cis retinal, a vitamin A analog, and the intradiscal region, which is important for the correct in vivo folding of the molecule. [1]

To date, there are more than 90 known rhodopsin mutations associated with autosomal dominant retinitis pigmentosa (ADRP); together, these mutations account for 30% of all ADRP patients.[2] The vast majority of rhodopsin mutations associated with ADRP are single-point mutations, distributed throughout the three domains of the protein, that result in single amino acid substitutions. There are also mutations that result in the deletion of amino acids as well.[2,3] Some sites have multiple substitutions, behaving as mutational "hot spots." There appears to be some correlation between the location of the mutation and the clinical severity of disease; for, example the cytoplasmic mutation (Pro347Leu) may result in a more severe clinical form of retinitis pigmentosa.[3]

The mutant that Dr Tso studied is found in the long cytoplasmic tail, which contains multiple threonines and serines that serve as phosphorylation sites. The role of this tail in the assembly and transport of rhodopsin to the outer segment is largely unknown.

Biochemically, the ADRP mutant rhodopsins fall into 3 distinct phenotypic categories[4]:

1. Some of the mutants bind and activate transducin inefficiently, even though the mutants are synthesized in normal amounts and bind 11-cis retinal similar to normal rhodopsin. Moreover, they show normal molecular structure and are found at the cell surface.

2. Most of the mutant rhodopsins are synthesized at low levels and bind 11-cis retinal poorly or not at all. In these mutants, the mutations are localized especially in the intradiscal and transmembrane regions. The altered function of the mutants may result from misfolding of a significant fraction of the protein.

3. An abnormal molecular structure of the mutant rhodopsin may prevent transport of the rhodopsin molecules to the cell surface, and the mutant rhodopsin molecules may be retained in the endoplasmic reticu-

lum, which can recognize and trap misfolded proteins. Retention of such proteins in the endoplasmic reticulum may lead to apoptosis.

I would like to ask Dr Tso and his colleagues several questions based on their morphologic studies. Do they have any evidence to suggest the ultrastructural site in rods that initiates the apoptotic death? In particular, did they find any initial changes in the endoplasmic reticulum of the rod cells? In the model they studied, were there any retinal vascular changes such as attenuation, especially in the animals at age 4 weeks? Are there any characteristic ultrastructural findings that could shed some light on the pathogenesis of cone cell degeneration in such a rhodopsin mutant model?

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JOHN T. FLYNN, MD. I would like to thank Dr Tso for opening up this area of an animal model of retinitis pigmentosa. I have a question for him. As I gather, this retinitis pigmentosa is very foreshortened in time as opposed to the disease in the human. Did you observe clinically, when you looked in with the ophthalmoscope, that the disease started in the mid-periphery and advance centripetally? The second part of my question is, is the distribution of rods and cones in the pig retina of the same character as it is in the human with the maximum number of rods outside of the fovea-macula region? How similar is this distribution to what we see in the human?

HUNTER L. LITTLE, MD. I would like to compliment Dr Tso on his typically beautiful paper. In view of experimental work on retinal cell transplantion, I wonder if it should be performed before the final stage of the disease. During the past 3 years, my younger daughter has been working on RPE cell transplantation with Professor M. del Cerro at the University of Rochester. They work with a group of rats that develop an aggressive retinitis pigmentosa beginning shortly after birth. The rats have a normal appearing retina initially, but within 6 months they develop a typical picture of retinitis pigmentosa with loss of photoreceptors. However, following early retinal pigment epithelium transplant, they found preservation of normal neurosensory retina in the areas of the RPE cell transplant. If the problem resides in the neurosensory retina why is it preserved by RPE cell

transplantation? Do you think the RPE cell and/or neurosensory retinal cell transplantation will be applicable in treatment of retinitis pigmentosa? If so, why wait until the very end stage of the disease?

ALAN FRIEDMAN, MD. I very much enjoyed this paper Mark. I have just one question. Is the process light triggered?

MARK TSO, MD. I would like to thank the discussants for their insightful comments.

I much appreciate the 3 piercing questions of Dr Rao on the morphologic clues of pathogenetic mechanisms of rod and cone degeneration in retinitis pigmentosa of the transgenic pig. In the pig model of retinitis pigmentosa as in other animal models and human patients, apoptosis was one of the pathogenetic pathways of cell death. In the pig retina, the initial changes in the degenerating rod cells appeared in the nuclei of the cells while the endoplasmic reticulum appeared unremarkable. Our study did not yet provide clues on how the mutant rhodospin leads to degeneration of the rod cells.

While retinal vascular changes are characteristically seen in human patients with retinitis pigmentosa, retinal vascular changes are not apparent in the transgenic pigs. However, the degenerative changes of the photoreceptor cells in the pig model occurred relatively rapidly at a young age. It is not surprising that retinal vascular changes were not observed, as these were most probably secondary changes to the retinal atrophy resulting from the degeneration of the neuronal elements.

The degenerative changes of the cone cells were most interesting. We observed densification of the cone cytroplasm simulating those observed in "delay neuronal cell death" secondary to excitotoxicity. As a result, we hypothesize that the excitatory amino-acids released from the degenerating rod cells might be one of the pathogenetic factors of cone degeneration. The degeneration of the cone cells took place at a very narrow window of time following the massive degeneration of rod cells. We are hopeful that during this short window period, photoreceptor rescuers such as the glutamate inhibitors might prevent the cone degeneration.

As to the question of Dr Alan Friedman, the retina of the pig was exposed to light since both pigs were allowed to freely roam in the lighted pigpen. It is difficult to speculate whether the rod cell death might be triggered by light.

However, Dr Elliot Berson showed that opaque corneal contact lens on human patients with retinitis pigmentosa did not delay the degeneration of the rod cells. On the other hand, in a recent study of transgenic mice that expressed mutant rhodospin in our laboratory, light exposure accelerated the degeneration of the photoreceptor cells when compared

with transgenic animals reared in cyclic light.[1]

I wish to thank the Program Committee for giving me an opportunity to share with you this investigative work.

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