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The Effect of Oxygen on Retinal Degeneration in Wild-type and *Hsp70.1* Knockout Neonatal Retinal Degeneration Mice

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The present study examined the effect of oxygen on photoreceptor degeneration in the retina of heat shock protein 70.1 (*hsp70.1*) knockout type and wild-type retinal degeneration (*rd*) mice. All the neonatal *rd* mice were exposed to hyperoixa for 5 days after birth, and then were returned to room air before being sacrificed. At the postnatal 10, 14, 18, and 21 days, the ratio of outer nuclear layer (ONL) thickness to total retinal thickness was compared between *hsp70.1* knockout type and wild type. The retina was also examined for DNA fragmentation by TdT-mediated biotin-dUTP nick-end labeling (TUNEL). In *hsp70.1* knockout type, the ratio of ONL to total retinal thickness was higher than that in the wild type at each time. There was the remarkable difference in the number and distribution of TUNEL-positive cells between *hsp70.1* knockout type and wild type *rd* mice. In conclusion, an oxygen-induced modulation of the rate of photoreceptor degeneration was more marked in the *hsp70.1* knockout type than wild type rd mice.

Key words: heat shock protein, knockout, retinal degeneration mice, oxygen, apoptosis, TUNEL

INTRODUCTION

It is well known that heat shock protein (Hsp) is induced by hyperthermia.[1-4] Induction of Hsp synthesis correlated with the time when photoreceptors were protected from light-induced damage.[4] Additional study indicated that the photoreceptor cell layer was the primary site of synthesis of 70-kDa Hsp(Hsp70) in the retina.[1] Interestingly, the level of the Hsp70 can be increased by the onset of retinal degeneration in the Royal College of Surgerons (RCS) rat with dystrophy.[5] However, the rise in ocular Hsp70 was insufficient to prevent progression of the degeneration.[5]

We have recently shown that a exposure of neonatal retinal degeneration (*rd*) mice to hyperoxia(80%) for 5 days can delay the degeneration of photoreceptor.[6] This experiment suggested that the protective effect of hyperoxia was associated with

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the enhanced synthesis of Hsps or neurotrophic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF). The neuroprotective effect of induced Hsp by sublethal hypoxia has been reported for cultured retinal ganglion cells.[2] If Hsp expression was disturbed in vivo, inhibition of apoptosis would be affected and the response to hyperoxia would be deteriorated in the retina. To the best of our knowledge, no previous studies have reported the change of apoptosis in hsp70 knockout animals.

To assess the role of Hsp70 in responding of photoreceptor from hyperoxia, we have prepared *hsp70.1* knockout *rd* mice and have compared their photoreceptor degeneration with that of wild-type *rd* mice.

METHODS

All procedures concerning animals were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. FVB/*rd* mice were obtained from Jackson laboratory (Maine, USA). Adult mice homozygous for the *hsp70.1* knockout were derived from matings after fourth generation back-crosses into the FVB/rd background.

Pups and mothers were housed in an acryl chamber within 12 h of birth, and oxygen delivered to the chamber and regulated by an oxygen analyzer (OM25ME, Ceramatec, Salt Lake city, UT, USA) to maintain an 80% environment for 5 days. And then the mice were placed with their mothers in room air. Light was cycled on a 12 h on, 12 h off schedule.

At the postnatal 10, 14, 18, and 21 days, all animals were sacrificed with intraperitoneal injection of phenobarbital (60 mg/kg). The eyes were enucleated and fixed in 4 % paraformaldehyde after incision was performed in the superior cornea to identify orientation of eyeball. All eyes were embedded in paraffin, and 4 μm thick histologic sections were taken along the vertical meridian from optic disc to superior cornea. The ratio of total retinal thickness and outer nuclear layer (ONL) thickness were measured with light microscopy (magnification $\times$400).

For TdT-mediated biotin-dUTP nick-end labeling (TUNEL) analysis by use of an in situ apoptosis detection kit (ApopTag; Intergen, NY, USA), deparaffinization was performed. After incubating with proteinase K, endogenous peroxidase was inactivated by incubating sections with 2% H_2O_2 for 5 minutes. Sections were preincubated in the equilibration buffer (provided in the kit) for 20 minutes at room temperature and were then treated with terminal deoxynucleotidyl transferase and digoxigenin deoxyuridine triphosphate for 1 h at 37°C. They were rinsed in buffer provided in the kit (stop/wash buffer) for 30 minutes at 37°C. Tissues were incubated with a peroxidase-coupled antidigoxigenin antibody for 30 minutes at room temperature. The 3'-OH DNA tail was detected by incubating retinas with a diaminobenzidine-H2O2 solution and counterstained with methyl green for 3 minutes. TUNEL-positive cells were counted in entire inner nuclear layer (INL) and ONL of all sections with light microscopy (magnification $\times$400).

The unpaired two-tailed Student's t-test was used to compare the ratio of ONL and to compare the number of TUNEL-positive cells in *hsp70.1* knockout type and wild type rd mice exposed to hyperoxia.

RESULTS

In our histologic study, the ONL was approximately 10- to 12-nuclei thick with well-formed inner and outer segments of the photoreceptor cells and a developed outer plexiform layer in *hsp70.1* knockout type as well as wild type *rd* mice. However, there was difference of change in the ONL thickness with aging between *hsp70.1* knockout *rd* mice and wild type *rd* mice from 10 to 21 days of age (Fig. 1, Table 1). The wild type *rd* mice retina showed a loss of photoreceptor cells, with a reduction of the ONL to one row of photoreceptor nuclei at postnatal 21 days. On the contrary, retina showed a remarkable preservation of many photoreceptors in *hsp70.1* knockout type rd mice (Fig. 1, Table 1)

The TUNEL method specifically labeled individual apoptotic nuclei in inner nuclear layer and ONL of the retina in *hsp70.1* knockout type as well as wild type rd mice. Most of the labeled nuclei were stained with a ring configuration and TUNEL-positive cells always appeared surrounded by unstained cells in the retina (Fig. 2). However, there was sig-

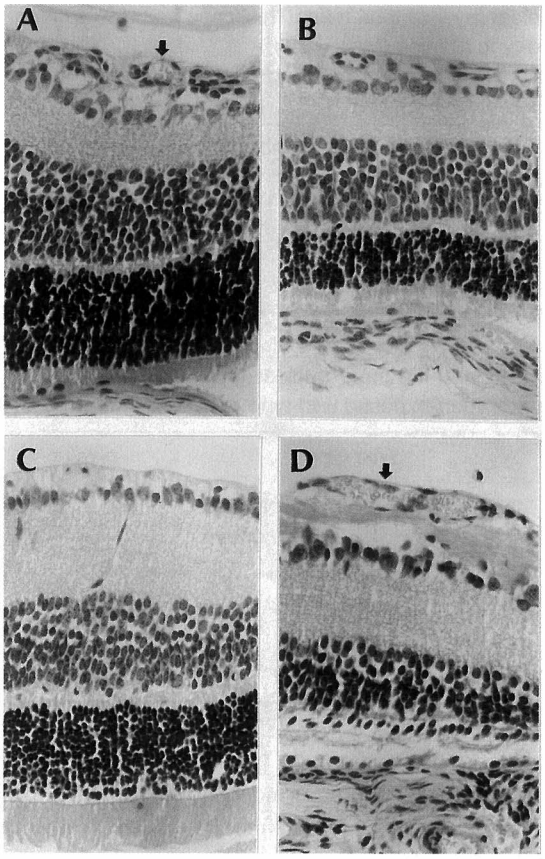

Fig. 1. Light micrographs of hematoxylin-eosin stained thin sections of mouse retinas at postnatal day 10 (A, C) and postnatal day 21 (B, D) after oxygen exposure during postnatal 5 days. A,B: *hsp70.1* knockout *rd* mice, C,D: wild type *rd* mice. There is no significant difference in the ratio of outer nuclear layer thickness to total retinal thickness between two retinas (A, C). The outer nuclear layer of B consists of several rows of nuclei, whereas the outer nuclear layer of D consists of only single row of nuclei. Additionally, extraretinal neovasuclarization (arrow) is demonstrated as abnormal vessel above the internal limiting membrane (original magnification: × 400).

nificant difference in distribution and number of TUNEL-positive cells between *hsp70.1* knockout type and wild type (Table 2). In *hsp70.1* knockout type rd mice, many TUNEL-positive cells were observed in INL at postnatal 10 days and in ONL at postnatal 14 days. There was no labeled cell in the retina at postnatal day 18 and 21.

In the wild type *rd* mice, many TUNEL-positive cells were present in ONL while a few cells were positive in INL at postnatal day 10 (Fig. 2). The number of TUNEL-positive cells in ONL had decreased with aging. However, a great majority of TUNEL-positive cells was showed in the ONL of the wild type *rd* mice at each time point (Fig. 2). By 14 to 21 days of age, a few TUNEL-positive cells were seen in INL.

DISCUSSION

Mice homozygous for the *rd* mutation display hereditary retinal degeneration and the classic rd lines serve as a model for human retinitis pigmentosa.[7,8] In *rd* mice, the rod population decreases from postnatal day 5 and degeneration is rapid-by postnatal day 17.[9,10] Apoptosis has been known as the mechanism through which photoreceptors die during normal mouse development as well as in several animal model of retinitis pigmentosa.[8,9,11,12] However, it remains unclear how apoptosis is triggered by a genetic defect in the beta subunit of the phosphodiesterase enzyme.

Hsps have been shown to act as an inhibitor of apoptosis.[13,14] As an antiapoptotic agent, Hsp70 mediates cellular protection by inhibiting the activation of effector caspases.[14,15] Recently, Kent and Tytell showed that intravitreally injected Hsp70 is taken up by the rat retinal cells and has the effect to protect photoreceptor from light damage.[13] Heat shock cognate(Hsc)70 mRNA levels declined during normal aging of the human retina, suggesting that decreased Hsc70 levels may contribute to the increased susceptibility of the retina to retinal disease.[16] We therefore hypothesized that *hsp70.1* knockout would change the development of photoreceptor degeneration and the response to hyperoxia in photoreceptor of *rd* mice. This study was performed to evaluate the difference in the effect of oxygen on photoreceptor degeneration between the retina of *hsp70.1* knockout type and that of wild-type *rd* mice. We believe this is the first study of apoptotic cell death during postnatal development in *hsp70.1* knockout *rd* mice.

In this study, an oxygen-exposed wild type rd mice retina showed the loss of photoreceptor cells,

Table 1. Comparison of the ratio of thickness of outer nuclear layer to total retinal thickness between oxygen-exposed hsp70.1 knockout mice and oxygen-exposed wild-type rd mice (Mean±SD)

Postnatal days	*hsp70.1* knockout type		Wild type		*p*-value
	Ratio(%)	Number of eyes	Ratio(%)	Number of eyes	
10	43.8±4.01	11	30.1±4.10	10	0.001
14	36.2±0.62	10	24.3±5.57	9	0.004
18	31.5±4.99	13	12.3±3.44	12	0.000
21	28.4 ±11.8	10	5.83 ±1.92	12	0.000

Table 2. Comparison of the number of TUNEL-positive cells in entire inner nuclear layer and outer nuclear layer between oxygen-exposed *hsp70.1* knockout mice and oxygen-exposed wild-type *rd* mice (Mean±SD)

Postnatal days	*Hsp70.1* knockout type			Wild type			*p*-value	
	INL	ONL	Number of eyes	INL	ONL	Number of eyes	INL	ONL
10	73.50±38.89	1.50±2.12	6	11.0±6.26	115.81±51.18	7	0.003	0.000
14	28.50±10.51	145.50±19.09	8	2.67±2.08	58.67±11.55	6	0.000	0.000
18	0	0	10	2.01±1.15	19.50±9.95	8	0.000	0.000
21	0	0	7	2.12±0.98	17.01±5.23	9	0.000	0.000

INL: inner nuclear layer, ONL: outer nuclear layer

with a reduction of the ONL to two or three rows of photoreceptor nuclei at postnatal 18 days and to single row of photoreceptor nuclei at postnatal 21 days. The time course of photoreceptor degeneration was delayed with oxygen, in agreement with the previous report.[6]

As demonstrated in Figure 2, extensive TUNEL staining was observed at postnatal day 10, mostly in cells of the ONL, in oxygen-exposed wild type *rd* mice. This finding corresponds with the report that apoptosis occurs during the early part of life at which time photoreceptor degeneration starts.[10] Lolley et al. also reported DNA loss from developing *rd/rd* retinas is maximal between 10 and 15 postnatal days.[9] The number of TUNEL-positive cells diminished with age, and stained cells were observed only occasionally at postnatal day 18 and 21. There were only a few stained cells in the INL at postnatal day 10 and nearly absent at thereafter. This observation is not consistent with the findings on apoptosis in rd mice observed by Chang et al.[11] They demonstrated that nuclei stained in both the INL and ONL at postnatal day 12, and extensive TUNEL staining continued in the ONL until postnatal day 21.[11] Although no data that could allow identification of the exact mechanism are available, the effect of oxygen may be certain possibilities in the modulation of apoptosis in INL as well as ONL.

Although the ratio of ONL thickness to total retinal thickness was decreased with aging, the preservation of photoreceptor was remarkable in oxygen exposed *hsp70.1* knockout type *rd* mice until postnatal 21 days. This result could not be expected because Hsp has been well known as protector against external stress.

The incidence of TUNEL-positive cells was high in the ONL of the retina in oxygen exposed *hsp70.1* knockout *rd* mice at postnatal day 14 while there was few or no stained cells at other times. This is consistent with an earlier report that the development of photoreceptor degeneration in the retina happened within approximately 2 weeks.[9,10] In contrast, INL staining was marked at postnatal 10 days. In normal mice, apoptosis was demonstrated at postnatal day 7, mostly in cells of the inner nuclear layer and the number of stained nuclei was diminished with age.[11] The time course of INL staining is consistent with our results.

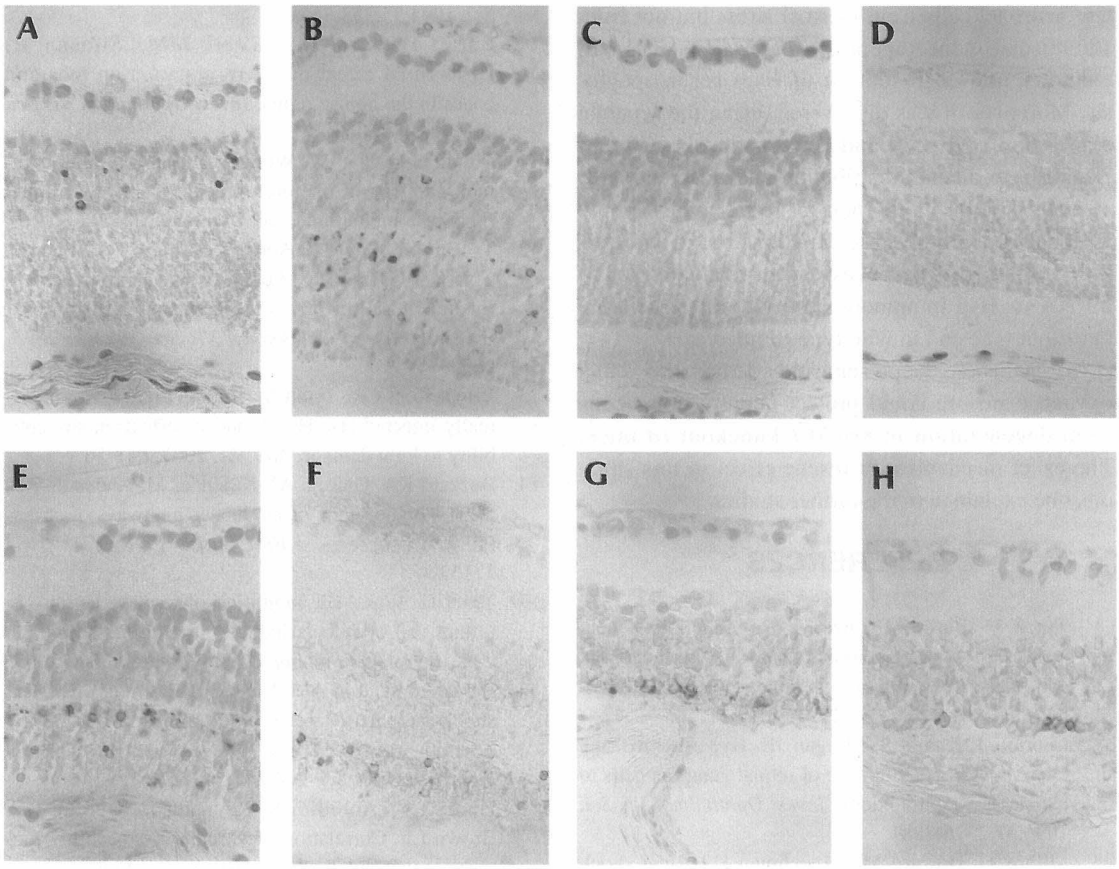

Fig. 2. TUNEL staining of *hsp70.1* knockout *rd* mice (A-D) and wild type *rd* mice (E-H) from postnatal 10 days to 21 days after oxygen exposure during postnatal 5 days (A, E: postnatal 10 days, B, F: postnatal 14 days, C, G: postnatal 18 days, D, H: postnatal 21 days). The labeled nuclei were stained with a ring configuration and TUNEL-positive cells always appeared surrounded by unstained cells in the retina.

We wonder that *hsp70.1* knockout rd mice show more protective effect by oxygen exposure than wild type *rd* mice. The reason that the survival of photoreceptor cells in *hsp70.1* knockout mice is unclear. However, several possibilities may be considered before making the conclusion. The first possible cause is exaggerated function or induction of neurotrophic factor induced by an oxygen exposure in *hsp70.1* knockout condition. The neurotrophic factors, including VEGF were induced in ischemic retina by hyperoxia,[17-19] and their protective effect on retinal degeneration was previously reported by several animal studies.[20-22] In RCS rats with inherited retinal dystrophy, intravitreal injection of FGF delayed retinal degeneration.[23] We reported that intravitreal injection of basic FGF resulted in extensive rescue of photoreceptors in *rd* mice for 18 days after injection.[24] We have also recently shown that a exposure of neonatal *rd* mice to hyperoxia (80%) for 5 days can delay the degeneration of photoreceptor.[6] However, to define clearly the role of neurotrophic factor, the protective effect of various survival factors have to be directly demonstrated in *hsp70.1* knockout rd mice.

The second possible explanation is that *hsp70.3* or other form of Hsps may be up-regulated or induced by an external stress with hyperoxia in *hsp70.1* knockout condition in mice. Mailhos et al. reported that the constitutive overexpression of either of the major Hsps, Hsp90 or Hsp70, can pro-

tect neuronal cells from thermal stress but not from stimuli that induce apoptosis.[3] Therefore, it is still unknown how each subtype of Hsps act in apoptosis. Moreover, it was discovered, using the separate expression of Hsc70 and Hsp70, that Hsc70 and Hsp70 have distinct distribution and different stage of expression in the normal rat retina, which imply regional and cell-specific functions.[25,26] Thus, further studies will be necessary to evaluate Hsp70 mRNA or Hsp immunoreactivity, both in *hsp70.1* knockout type and in wild type *rd* mice.

In conclusion, the present study demonstrated that oxygen exposure could protect photoreceptor cells from degeneration in *hsp70.1* knockout *rd* mice. The exact mechanism of rescue effect in this study must be explained in the further studies.

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