Light-Induced Apoptosis in the Neonatal Mouse Retina and Superior Colliculus

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PURPOSE. Apoptosis occurs naturally in the rodent retina and superior colliculus (SC) during the neonatal period. The authors used mice to demonstrate the dependency of this apoptosis on the light stimulation and the developmental period.

METHODS. A number of apoptotic cells were counted in the retina and SC from a group of newborn mice reared in constant darkness (DD group), a group reared in normal light and dark conditions (LD group), and a group reared in constant darkness up to P7 and then transferred to normal condition (DD-to-LD group). Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick end labeling (TUNEL) was used for visualization of the apoptotic cells.

RESULTS. In the LD group, apoptotic cells significantly increased in the retinal nuclear layers, including both the outer and inner nuclear layers, the retinal ganglion cell layer, and SC at postnatal day 1 (P1) and postnatal day 2 (P2). The number of apoptotic cells in the ganglion cell layer and SC reached the maximum level at P1. In contrast, in the DD group, an increase in the number of apoptotic cells was not observed. At P9, no significant increase in the number of apoptotic cells was observed in the outer nuclear layer, ganglion cell layer, and SC either in the LD, DD, or DD-to-LD groups, but the LD and DD-to-LD groups showed a significant increase in the inner nuclear layer compared to the DD group.

CONCLUSIONS. Apoptosis during the neonatal period in the mouse visual system is induced by a light stimulus. This apoptosis was not induced after P7 in the retinal ganglion cell layer and SC, even if excessive cells survived. (Invest Ophthalmol Vis Sci. 1999;40:3079–3083)

Irreversible damage due to degeneration of retinal visual cells is caused by exposure to intermittent and constant strong light.1,2 This degeneration is thought to proceed by apoptosis.3 Rats reared in ordinary cyclic dim-light conditions exhibit lower rhodopsin levels in photoreceptor cells than those reared in constant darkness.5 These facts suggest that light stimulation influences the survival of visual cells in the retina. In addition to the apoptosis that can develop under abnormal conditions, apoptosis also is induced in the normal developing visual system. Studies have shown that apoptotic cells dramatically increase after birth in mouse neonatal retina4 and rat neonatal superior colliculus (SC).5

Generally, apoptosis during development is necessary to form functionally normal tissue by eliminating unnecessary excessive cells. In the nervous system, naturally occurring neuronal cell death (apoptosis) during the neonatal period is important for the formation of appropriate neuronal circuits by elimination of neurons with erroneous connections.6 What kinds of stimuli distinguish necessary neurons from unnecessary ones? In the developing visual system, the evidence suggests that one stimulus that might is outside light. We demonstrate here that exposure to light is necessary to induce apoptosis in the mouse retina and SC during the neonatal period, but this apoptosis is not induced 1 week after birth, even if excessive neurons have not been removed.

MATERIALS AND METHODS

Experimental Animals

We used pregnant ddY albino mice from our breeding colony. All animals were cared for and used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The day after mating was defined as E0 and the day of birth as P0, which corresponded to E19. Mice were divided into three groups. Pregnant mice were transferred to the dark room at E18, and dams and neonates were reared in complete darkness until sacrifice (DD group). As a control, dams and neonates were maintained on a daily cycle of 12 hours light and 12 hours darkness (LD group). The lights were on from 8 AM to 8 PM. Illumination was provided by white fluorescent lamps (40 W, ×6). The mean distance between the lamps and cages was approximately 2 m. Light intensity in the cages ranged from 200 to 250 lux. Dams and neonates reared in the dark room until P7 were transferred to the normal conditions (LD 12:12) and maintained for 2 days (DD-to-LD group).
Preparation of Tissue Sections
Newborn mice at P0 (=E19), P1, P2, P9, P14, and P21 were overdosed with pentobarbital and fixed by perfusing 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) through their hearts. The brains were cut in half sagittally, the eyeballs were removed, and then both the brains and the eyeballs were immersed in the 4% paraformaldehyde solution at 4°C for 2 days. E18 fetuses taken from pregnant mice anesthetized with an overdose of pentobarbital were briefly rinsed with saline and immersed in the fixing solution at 4°C for 2 days. After washing with phosphate-buffered saline (PBS), tissues were immersed in 20% sucrose in PBS overnight at 4°C and frozen with dry ice. Cross sections (10 μm) were cut by a Cryostat and stuck on glass slides coated with 0.01% poly-l-lysine. Retina sections cut parallel along the optic axis within approximately 0.2 mm from optic nerve were used for counting.

Terminal Deoxynucleotidyl Transferase-Mediated Biotin-dUTP Nick-End Labeling
Sections were immersed in 0.3% Triton X-100 in 0.05 M Tris-HCl, pH 7.5, at room temperature for 1 hour and incubated with 1.5 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) for 15 minutes at 37°C. After precipitation in a terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, and 1mM cobalt chloride) for 10 minutes, sections were incubated in a TdT buffer containing 50 units/ml TdT (Takara, Tokyo, Japan) and 0.2 mM biotinylated 16-dUTP (Boehringer Mannheim) in a humid atmosphere for 2 hours at 37°C. After washing with PBS, endogenous peroxidase was inactivated by incubating in 3% H2O2 and 0.1% sodium azide for 10 minutes at room temperature. Sections were incubated with Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) for 2 hours at room temperature and stained with 3,3’-diaminobenzidine, H2O2, and ammonium nickel(II) sulfate hexahydrate as substrate. Each section was counterstained with neutral red.

Statistical Analyses
Sections including entire areas of the retina and SC at each time point were photographed. From these data, numbers of terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL)-positive cells/1000 cells were calculated. Data were analyzed by analysis of variance (ANOVA) with Scheffe’s F test.

RESULTS
Influence of Light Stimuli on the Induction of Apoptosis in the Neonatal Mouse Visual System
Figure 1 shows representative photographs of TUNEL-positive cells in the neonatal retina and SC at P1. Deep dark staining of TUNEL-positive cell nuclei was seen in the outer and inner nuclear layers (ONL and INL), the retinal ganglion cell layer (GCL), and the SC from the LD group (Figs. 1A, 1C). In the DD group, however, the number of TUNEL-positive cells was smaller (Figs. 1B, 1D). We performed a quantitative analysis for a temporal change in number of TUNEL-positive cells in the retina and SC during the neonatal period. In the early neonatal period, when the boundary between the ONL and INL is unclear, we did not distinguish between these two layers in counting apoptotic cells. The nuclei of the photoreceptor cells were included in the ONL and the nuclei of amacrine cells, bipolar cells, horizontal cells, and Müller glia were included in the INL. However, classification of cell types was not performed in this experiment. Before birth (E18), few TUNEL-positive cells were seen in the ONL and INL (0.4 ± 0.3/1000 cells) (Fig. 2A). After birth, a small increase in the number of TUNEL-positive cells was observed at P0 (1.1 ± 0.1). The number of apoptotic cells clearly increased at P1 (2.8 ± 1.5, P < 0.02) and was even greater at P2 (3.4 ± 1.4, P < 0.01). In contrast, the number of TUNEL-positive cells in the DD group was quite low, nearly equal to the level at E18.

In the retinal GCL, ganglionic neurons with large nuclei are easily identified. The number of apoptotic ganglion cells was small before exposure to light at E18 (1.5 ± 1.3). After a small increase at P0, apoptotic cells dramatically increased at P1 (17.9 ± 6.9, P < 0.01), but then decreased at P2 (9.6 ± 4.2, P < 0.06). The number of apoptotic ganglion cells in the DD group did not increase from E18 to P0. At P14 and P21, there was no apoptotic cell in the retina, in either the LD or DD group.

In the SC, the superordinate center of the retina, the temporal change in number of neonatal apoptotic cells resembled that of retinal ganglion cells. Although few TUNEL-positive cells were found at E18 (0.4 ± 0.5) in the LD group, a significant increase in apoptotic cells was observed at P0 (4.4 ± 1.5, P < 0.05), the number of apoptotic cells reached the maximum level at P1 (8.7 ± 3.4, P < 0.01), and then decreased at P2 (5.0 ± 2.2, P < 0.02). In the DD group, no significant increase in apoptotic cells was found from E18 to P2, as in the retina. To compare with the SC, we also analyzed the occurrence of apoptosis during the neonatal period in the inferior colliculus. Although few apoptotic cells were found at E18 as in the other portion of brain, there was a significant increase in apoptotic cells at P0 in both the LD and DD groups. This is a reasonable result because the inferior colliculus is part of the auditory system, and breeding in the darkness did not eliminate outside sounds.

Existence of a Critical Period for Light-Induced Apoptosis in the Neonatal Mouse Visual System
To investigate whether the potency of light-induced apoptosis is retained until a much later period in the development of the mouse visual system, we performed the following experiments. Mice bred in constant darkness until P7 were transferred and maintained under normal conditions up to P9 (DD-to-LD group). The number of TUNEL-positive cells was counted in the ONL, INL, GCL, and SC in P9 mice from the LD, DD, and DD-to-LD groups. Since the boundary between the ONL and INL is clear at this stage, we distinguished between these two areas.

In the ONL, TUNEL-positive cells were scarcely observed in any of the three groups. The number of TUNEL-positive cells was at a low level in each group. In the LD and DD-to-LD groups, the numbers in the GCL and SC were almost at the same level. Although the DD group showed lower values, there was no significant difference between the three groups. These results suggest that light fails to induce apoptosis in these areas, even if excessive cells survive by escaping from apoptosis that occurs during the early neonatal period. In contrast, the numbers of TUNEL-positive cells in the INL of the LD and DD-to-LD groups were significantly higher than those in the
DD group ($P < 0.05$ and $P < 0.01$, respectively). Cells in the INL retain susceptibility to light for the induction of apoptosis. Mice from the LD and DD groups were maintained until P14 and P21. No apoptotic cells were found either in the retina or SC at these periods. No morphologic change was observed in the retina and SC of DD group mice.

**DISCUSSION**

In the nervous system, neurons were excessively generated during the early stages of development and were all eliminated by apoptosis, except for those with appropriate functions in the course of development. It is believed that the selection by apoptosis is genetically programmed. But, we have shown here that light triggers the induction of apoptosis in the mouse retina and SC during the early postnatal period. This suggests some type of apoptosis depends on outside stimuli.

The peak of apoptosis in retinal ganglion cells and neurons of the SC from the LD group mice was at P1. To date, developmental apoptosis observed as pyknotic cells has been reported, under normal conditions, in the mammalian visual system. Apoptosis declined after reaching a peak at P2 in the mouse retinal ganglion cells and at P0 in the rat SC. The patterns of temporal change of apoptosis in the LD group were more or less consistent with these cases. Because proliferation of these neurons terminates before birth, their apoptosis is independent of cell cycle progression. Apoptosis was observed in the ONL and INL during later developmental periods up to at least P9; however, no apoptotic cells were found in any area at P14 and P21. In addition, the population of apoptotic cells in the retina was considerably larger than indicated by previously reported data. We presume that this may be because TUNEL shows a higher sensitivity for detection of apoptosis than light-micrographic observation of pyknotic cells.

During the early neonatal period in rats and mice, the eyes are not open, the retinas are immature, and the outer segment of photoreceptor cells is not formed. So, which cells and molecules transmit information from light stimuli to induce apoptosis during the early neonatal period? Rhodopsin is immunohistochemically detected in immature rod photoreceptor cells with no outer segments in rat retinas at P3. Several monoclonal antibodies against rhodopsin show immunohistochemical staining in cell bodies of immature neuroblast-like cells positioned in the neuroblastic zone beside the pigmented epithelium even in the P1 rat retina. These cells seem to be immature photoreceptor cells. Opsin mRNA is expressed in P1
rat retinas at low but detectable levels. Developmental changes in mouse retinas may be similar to those seen in rat retinas. This evidence suggests that rhodopsin exists in immature rod photoreceptor cells even during the early neonatal period. We presume that these immature photoreceptor cells may possess the light sensitivity, even though levels of rhodopsin are low and rhodopsin-expressing cells are much more rare in neonates than in adults.

In rats and hamsters, the SC receives the projection from both contralateral and ipsilateral eyes during the neonatal period. During 2 weeks after birth, most of the projection from the ipsilateral eye is reduced and restricted to the rostromedial SC. This restriction is prevented by contralateral eye enucleation and by injection of tetrodotoxin into the contralateral eye to inhibit neuronal activity during the neonatal period. This process is due to apoptosis of ipsilaterally projecting retinal ganglion cells rather than to removal of ipsilaterally projecting axons from ganglion cells with bifurcating axons. These results suggest that light-induced apoptosis occurs preferentially in ganglion cells with ipsilateral retinocollicular projection. It is still unknown, however, why contralateral projection always wins the game and how this competition is regulated. Patterned visual input appears to be independent of this phenomenon because the eyes are still not open. The influence of the competition between these two projections on the induction of apoptosis in the SC is also unknown.

From our results with the DD-to-LD group, we determined that a critical period exists in the susceptibility to light for the induction of apoptosis. In retinal ganglion cells and neurons of the SC, in particular, light fails to induce apoptosis at P7, even when excessive neurons destined to die survive. Mouse optic nerves enter the SC several days before birth and axon branching and synapse formation proceed around P7. Because developmental change in apoptosis of the GCL and SC resemble each other without time lag, main pathways have already formed at birth. Since the timing of the loss of susceptibility to light-induced apoptosis is coincident with the period

**FIGURE 2.** Developmental change in the number of TUNEL-positive cells in the nuclear layer including the outer and inner nuclear layer (A), retinal ganglion cell layer (B), and superior (C) and inferior colliculi (D). Values represent the mean ± SD; n = 5 (animal number). *P < 0.005; **P < 0.05, relative to E18 by Scheffe’s F test.

**FIGURE 3.** TUNEL-positive cells in the LD, DD, and DD-to-LD groups at P9. A statistically significant difference was observed only in the INL. Values represent the mean ± SD; n = 5 (animal number). *P < 0.005; **P < 0.05, relative to INL of the DD group by Scheffe’s F test.
of synaptic reconstruction, stimulation through newly constructed synapses may prevent apoptosis.

Several factors related to the induction of apoptosis have been identified recently. In the nervous system, neuronal cell death under artificial and pathologic conditions is suppressed by antagonists of \(N\)-methyl-\(D\)-aspartate receptors\(^{17}\) and inhibitors of nitric oxide synthase.\(^{18,19}\) The induction mechanism of developmentally occurring neuronal cell death has not yet been elucidated; however, similar factors also may be included in this process.

Finally, the functional abnormality caused by a blockade of naturally occurring apoptosis in the visual system is not clearly understood. We suppose that DD group may show the neonatal type of retinocollicular projection even until adulthood. Because our animal model has no neural impairment derived from enucleation or neurotoxins, it may be used for physiologic examination of the influences of abnormal retinocollicular projection.

References