Iron-Induced Apoptosis in the Photoreceptor Cells of Rats

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PURPOSE. To determine whether apoptosis is involved in retinal degeneration induced by intravitreal implantation of 5 iron particles in rat eyes.

METHODS. Autoclaved iron particles were implanted in the vitreous cavities of the experimental eyes. Glass chips were implanted in the control eyes. The experimental eyes were enucleated at various time intervals from days 1 to 15. Retinal degeneration was examined using the TdT-mediated, dUTP-biotin nick-end labeling (TUNEL) method. Electrophoresis on agarose gel was used to detect internucleosomal DNA fragmentation.

RESULTS. TUNEL-positive nuclei were observed only in the outer nuclear layer beginning on day 2. The nuclei spread throughout the outer nuclear layer by the end of day 3. No TUNEL-positive nuclei were observed in other layers throughout the experimental period. Analysis of DNA, extracted from the retinas by electrophoresis on agarose gel, revealed a typical ladder pattern of internucleosomal DNA cleavage in the experimental eyes.

CONCLUSIONS. Apoptosis occurred during photoreceptor cell death at the early phase of iron-induced retinopathy in these rats. Like photic injury, iron-induced apoptosis was limited to the outer nuclear layer. (Invest Ophthalmol Vis Sci. 1998;39:631-633)

Siderotic retinopathy, siderotic cataract, and glaucoma secondary to siderosis are induced either by the presence of intraocular iron foreign bodies or intraocular hemorrhage. Many histologic investigations on intraocular iron toxicity were reported in the 1970s when necrosis was widely accepted as the principal mechanism of cell death. Therefore, "necrosis" was used to describe iron-induced retinopathy in earlier reports. Since Kerr et al. called attention to the distinction between apoptosis and necrosis, there have been increasing reports describing apoptosis in many pathologic conditions.

in recent years. Tso et al. described the involvement of apoptosis in photic injury to the retina.

Free-radical formation is a common factor in intraocular iron toxicity and photic injury to rat retina. A series of biochemical studies documented the role of free radicals by the oxidation of ferrous iron in siderotic retinopathy. Noell postulated that the free radical produced from photochemical reactions is an important factor in photic injury. The present objective is to determine whether apoptosis is also involved in the iron-induced retinopathy seen in photic injury.

MATERIALS AND METHODS

Rats were chosen for the present study so the results could be compared directly with those of our previous studies on photic injury. Forty-two male Sprague-Dawley rats 40 to 60 days of age were divided into two groups of 32 experimental animals and 10 control animals. The animals were fed ad libitum and maintained on a 12-hour light-dark cycle. The experimental conditions conformed to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Autoclaved, 5-mg iron particles (99.98% purity; Aldrich, Milwaukee, WI) or glass chips of similar size were used. An in situ apoptosis detection kit was supplied by Oncor (Apop-Tag; Gaithersburg, MD). Proteinase K and DNase-free RNase A were supplied by Sigma Chemical (St. Louis, MO). The DNA ladder marker was supplied by Life Technologies (Gaithersburg, MD).

Animals were anesthetized by intramuscular injections of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). Local analgesia was achieved with drops of 0.5% proparacaine. A limbal-based conjunctival flap, 3-mm wide, was made under an operating microscope (M840; Leica, Nussloch, Germany). One iron particle or glass chip was inserted into the vitreous cavity through a lesion at a position 1 mm posterior to the insertion of the superior rectus muscle. The foreign body was inserted into the vitreous cavity by a micropick monitored under the operating microscope. The conjunctival flap was closed by cautery. Ointment (Maxitrol, 00630; Alcon, Fort Worth, TX) was administered after the operation. Eye-drops (Maxitrol, 00630; Alcon) were given daily after ocular examination. Groups of four animals were euthanatized with CO2 at 24, 48, 60, and 72 hours and 4, 7, 10, and 15 days after implantation. For control, one glass chip was implanted in the vitreous cavity of each control animal in the same manner. Groups of two animals were euthanatized at 2, 4, 7, 10, and 15 days after surgery.

All eyes were enucleated and fixed in 10% buffered formaldehyde. Each eye was opened in an anterior-posterior direction. The iron particle was removed from the vitreous cavity of each treated eye after overnight fixation and embedded in paraffin.

Tissue sections were examined using the TdT-mediated, dUTP-biotin nick-end labeling (TUNEL) method. Retinas from Royal College of Surgeons (RCS) rats 35 days of age were used as a positive control for the TUNEL method. To distinguish TUNEL from background staining, an adjacent serial section of the retina was processed for TUNEL without the incubation with TdT. Methyl green was used for counterstaining.

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DNA extraction and DNA fragmentation analysis were carried out as previously described. The extracted retinal DNA was analyzed for internucleosomal cleavage by electrophoresis on a 2% agarose gel. The ladder pattern of DNA fragments was visualized through ultraviolet light after ethidium bromide staining and was photographed using the MP-4 system (Polaroid). Retinal DNA extracted from RCS rats 35 days of age and normal Sprague-Dawley rats was used as a positive and a negative control, respectively. Schemeltzer's method was used to examine the possible relationship between the apoptotic cell death and the location of iron deposit.

RESULTS
Detection of Apoptosis by the TUNEL Method
Nuclear DNA fragmentation was visualized by examining the brownish color of the TUNEL-positive nuclei (Fig. 1). There was no detectable TUNEL-positive nucleus on day 1 (Fig. 1A). TUNEL-positive nuclei became noticeable in the outer nuclear layer on day 2. The number of TUNEL-positive nuclei increased rapidly after day 2 (Figs. 1B, 1C, 1D). They are seen throughout the outer nuclear layer on day 2.5 (Fig. 1C). Giant cells with TUNEL-positive cytoplasm appeared on day 3 (Fig. 1D). There were no significant TUNEL-positive nuclei in the inner nuclear or ganglion cell layers throughout the time period chosen for this study.

There were no TUNEL-positive nuclei observed in the control eyes implanted with glass chips on days 2, 4, 7, 10, and 15. The negative result of one control eye (day 4) is shown in Figure 1E.

Visualization of Iron Deposit Using Schemeltzer's Method
A tissue section from a rat on day 4 after iron implantation was used. Despite massive cell loss in the outer nuclear layer, there was a negligible amount of iron deposit in the heavily damaged outer nuclear layer. Heavy iron deposit was observed in the peripheral vitreous cavity (Fig. 1F).
DNA Fragmentation Demonstrated by Electrophoresis

The ladder pattern of a mixture of standard DNA fragments having molecular sizes varying from 100 to 2072 bp is shown in Figure 2 (lane M). It is well known that internucleosomal DNA fragmentation occurs in the retina of RCS rats. Therefore, we used the retinal DNA from an RCS rat as a positive control to demonstrate the ladder pattern of the DNA isolated from a tissue with apoptotic cells (lane 1). There was no ladder pattern observed for retinal DNA from a normal rat (lane 2) on the day after iron implantation, when apoptosis had not begun (lane 9). The ladder pattern of DNA fragmentation was observed on days 2 and 3 (lanes 3 and 4, respectively) and coincided with the large number of TUNEL-positive nuclei shown in Figure 1. DNA fragmentation was not observed in the control eyes on days 2 and 4 after glass implantation (lanes 5 and 6, respectively). When the outer nuclear layer was completely destroyed on day 15, there was a smeared appearance in the location of the DNA of high molecular size (>2000 bp), yet no DNA fragment smaller than 1000 bp was observed (lane 7). There was no DNA fragmentation among the specimens prepared using the glass-implanted control retina on DNA fragmentation 15 days after glass implantation (lane 8).

DISCUSSION

The present data demonstrated the involvement of apoptosis in iron-induced retinopathy. The occurrence of apoptosis was demonstrated using the visualization of TUNEL-positive nuclei (Figs. 1B, 1C, 1D) and the electrophoresis that is characteristic of DNA fragments (Fig. 2). The negative result (Fig. 1E) of intraocular implantation of a glass chip rules out a nonspecific involvement of apoptosis in all intraocular trauma.

There is a difference in the time required for the onset of apoptosis after photic injury and that after intraocular iron implantation. Apoptosis occurred immediately after exposure to light. When iron was implanted in the vitreous cavity, there was a latent period of approximately 1 day. Scattered apoptotic cells in the outer nuclear layer were noticed on day 2 and quickly spread throughout the outer nuclear layer by day 3. The latent period required for the manifestation of iron toxicity is well known. It takes time for a solid iron particle to produce an effective soluble iron concentration, whereas photochemical reactions occur instantaneously as light enters the eye. Once a sufficient concentration of solubilized iron was formed, apoptosis spread throughout the outer nuclear layer rapidly.

The most interesting observation is the limitation of apoptosis to the outer nuclear layer just as in our previous observations on photic injury. Because iron was placed in the vitreous cavity, the solubilized iron diffused through the ganglion cell layer and the inner nuclear layer before it reached the outer nuclear layer. The majority of the solubilized iron remained in the peripheral vitreous (Fig. 1F). Therefore, the iron toxicity would be expected to begin in the ganglion cells and the inner nuclear layer before damaging the outer nuclear layer. The present result indicates that the outer nuclear layer is sensitive to free-radical damage. The occurrence of polyunsaturated fatty acids in the rod and cones is one of the possible factors affecting the sensitivity of the outer nuclear layer to free-radical damage.

Apoptosis occurs at the early stage of iron-induced retinopathy (days 2–3). The later appearance of giant cells indicates that necrosis occurs after the onset of apoptosis. The number of TUNEL-positive nuclei reached a peak with a noticeable thinning of the outer nuclear layer on day 3. Many giant cells filled with TUNEL-positive debris appeared in the outer nuclear layer and subretinal space (Fig. 1C) after day 3. Previous publications by others also reported the appearance of giant cells and described them as macrophages or phagocytosing retinal pigment epithelial cells.

References