Photoreceptor Apoptosis Induced by a Single Systemic Administration of N-Methyl-N-Nitrosourea in the Rat Retina

Motomaro Nakajima,*† Kenshi Yuge,*† Hideto Senzaki,† Nobuaki Shikata,† Hirohiko Miki,* Masanobu Uyama,* and Airo Tsubura†

From the Departments of Ophthalmology* and Pathology,† Kansai Medical University, Moriguchi, Osaka, Japan

Retinal degeneration was induced by a single intraperitoneal injection of N-methyl-N-nitrosourea in female Sprague-Dawley albino rats at 50 days of age by two dose regimens, which were observed sequentially at 24, 48, and 72 hours and 7, 21, and 35 days after the treatment. After a dose of 75 mg/kg, methylnitrosourea evoked progressive retinal degeneration in all treated rats whereas a dose of 50 mg/kg was less effective. The 75-mg/kg-treated rats showed selective destruction of the photoreceptor cells by an apoptotic mechanism, as confirmed morphologically and by the terminal dUTP nick end labeling method. Apoptosis had already started at 24 hours after the treatment and was completed by day 7. During the photoreceptor degeneration, proliferation of glial fibrillary acidic protein and vimentin-positive Müller cells as detected by proliferating cell nuclear antigen labeling appeared at 48 hours and was prominent 72 hours after the treatment, and macrophage infiltration within the retina as recognized by ED1 positivity was maximal 7 and 21 days after the treatment. Retinal degeneration was also induced in female Brown-Norway colored rats in a similar dose-dependent manner. Pigment epithelium was discontinuous above Bruch's membrane, and migration of the swollen pigment epithelium toward the inner nuclear layer was seen 7 days after the treatment. Therefore, as also confirmed electron microscopically, the most striking change was the destruction of photoreceptor cells by the apoptotic process, followed by Müller cell proliferation, pigment epithelium migration, and macrophage infiltration for cell debris phagocytosis, resulting in a thin remnant of retina with attenuated inner nuclear cells in direct contact with Bruch's membrane or with the pigment epithelium and/or with the Müller cells 35 days after the treatment. (Am J Pathol 1996, 148:631–641)

Retinitis pigmentosa is a human disease characterized by loss of photoreceptor function in which photoreceptor degeneration leads to visual loss and eventually to blindness. There are several hereditary animal models for retinitis pigmentosa that carry mutant genes that develop retinal degeneration. The eye is highly sensitive to toxic substances, and various chemicals are known to cause retinal toxicity. Among the chemicals, N-methyl-N-nitrosourea (MNU), a nitroso compound distributed widely in the environment, is a direct acting alkylating agent that does not require metabolic activation and is known to be a potent carcinogen, teratogen, and mutagen in laboratory animals. A single dose of MNU induces neoplasms in many animal species and in a variety of tissues. Despite the numerous publications, reports of ocular involvement after exposure to MNU have been limited. Intraocular melanomas are induced by a topical administration of MNU to rat eyes, but if DNA damage caused by alkylaion is severe, it will result in cell death. Toxicity and carcinogenicity may be independent events.

Herold first reported photoreceptor degeneration after systemic administration of MNU to Syrian hamsters. Later, photoreceptor degeneration was induced in rats and even transplacently to their offspring in mice. The degeneration was initially reported in eyes of rats autopsied 3 to 10 months

Supported in part (to A. T.) by the Science Research Promotion Fund of the Japanese Private School Promotion Foundation (1994). Accepted for publication November 1, 1995. Address reprint requests to Dr. Airo Tsubura, Department of Pathology, Kansai Medical University, Moriguchi, Osaka 570, Japan.
Table 1. Incidence and Severity of Retinal Lesions of S-D Rats Treated by a Single Intraperitoneal Administration of MNU

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Dosage (mg/kg)</th>
<th>Number of rats</th>
<th>Retinal pigmented epithelial cells, disorganization</th>
<th>Photoreceptor outer segment</th>
<th>Photoreceptor inner segment</th>
<th>Photoreceptor nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Disruption</td>
<td>Depletion</td>
<td>Disruption</td>
<td>Depletion</td>
</tr>
<tr>
<td>24 hours</td>
<td>75</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>48 hours</td>
<td>75</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>72 hours</td>
<td>75</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>7 days</td>
<td>75</td>
<td>4</td>
<td>4</td>
<td>—</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2</td>
<td>2</td>
<td>—</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>21 days</td>
<td>75</td>
<td>4</td>
<td>4</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>35 days</td>
<td>75</td>
<td>3</td>
<td>3</td>
<td>—</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>3</td>
<td>—</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are shown as number of rats affected.

after MNU treatment, but Ogino et al. reported that these lesions were completed within 5 weeks after MNU treatment. There are two forms of cell death, necrosis and apoptosis. In mutant animals with retinal degeneration, photoreceptor cell death occurs by an apoptotic mechanism. MNU may cause cell death through a necrotic mechanism or by apoptosis. In this study, the morphological characteristic of the development of retinal degeneration was examined after two regimens of a single intraperitoneal injection of MNU in Sprague-Dawley (S-D) albino rats and in Brown-Norway (B-N) colored rats. In situ labeling of apoptotic cells by terminal dUTP nick end labeling (TUNEL) was used to detect apoptosis-specific internucleosomal DNA fragmentation.

Materials and Methods

Animals

Sixty 6-week-old female albino S-D rats were purchased from CLEA (Osaka, Japan), and five female colored B-N rats were purchased from Seiwa Institute of Experimental Animals (Fukuoka, Japan). Animals were maintained on a basal diet, CMF (Oriental Yeast, Kyoto, Japan), and watered freely. Rats were housed in plastic cages with wood-chip bedding, two to five rats per cage, in an air-conditioned room, at 22 ± 2°C and a relative humidity of 60 ± 10%, with a 12-hour light/dark cycle. The illumination intensity was <60 lux at the cage level.

Chemical

MNU was obtained from Nacalai Tesque (Kyoto, Japan). Upon arrival, MNU was kept in −20°C in the dark. MNU solution was freshly prepared, dissolved in physiological saline containing 0.05% acetic acid, just before use.

Experimental Procedure

At 50 days of age, 33 S-D rats received a single intraperitoneal injection of MNU (50 mg/kg body weight), 23 S-D rats received 75 mg/kg MNU, and 4 S-D rats remained untreated. At 24, 48, and 72 hours and at 7, 21, and 35 days after MNU treatment, rats were anesthetized by ether and killed by cervical dislocation, and 4 untreated S-D rats were killed at 85 days of age. In addition, 3 B-N rats received 50 mg/kg MNU and 2 B-N rats received 75 mg/kg MNU and were killed 7 days after the treatment. After MNU treatment, all rats were weighed every day for a week and thereafter once a week. After the animals were killed, both eyes were quickly removed, and complete autopsies were done on all animals.

Tissue Fixation and Processing

In all cases, one eye was fixed overnight in 10% neutral buffered formalin; others were either fixed 4 to 6 hours in methacarn or 4 hours in 0.5X Karnovsky solution. After 1 hour in the 0.5X Karnovsky fixative, the eyes were bisected for better penetration of the fixative. The formalin- and methacarn-fixed tissues were routinely processed for paraffin embedding.
MNU-Induced Photoreceptor Apoptosis in Rats

AJP February 1996, Vol. 148, No. 2

**Figure 1.** Retina of untreated and 75-mg/kg MNU-treated S-D rats. a: Untreated control. 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. b: At 24 hours after the treatment. Note pyknosis and karyorrhexis of the photoreceptor cells. c and d: At 48 and 72 hours after the treatment, respectively. Destruction of the photoreceptor cells and photoreceptor segments is progressing. e: At 7 days after the treatment. Note photoreceptor cell depletion, but several layers of cells remain between inner nuclear layer and choroid. f and g: At 21 and 35 days after the treatment, respectively. Inner nuclear cells are in direct contact with choroid or leaving several layers of cells in between. H&E; magnification, ×300.

and stained with hematoxylin and eosin (H&E) or with the periodic acid Schiff (PAS) method. All other organs and tissues were dissected, weighed, fixed in 10% neutral buffered formalin, and processed for histological examination.

**TUNEL Method**

DNA fragmentation at the single-cell level was observed using TdT-mediated dUTP-digoxigenin nick end labeling, which stains nicked DNA ends by digoxigenin-labeled dUTP.17 Formalin-fixed tissues were applied for TUNEL staining using an in situ apoptosis detection kit (Apop-Tag, Oncor, Gaithersburg, MD) according to the manufacturer’s instructions. The peroxidase label was visualized with 3,3'-diaminobenzidine (Wako Pure Chemical, Osaka, Japan).

**Immunohistochemistry**

Serial sections were subjected to the immunohistochemical studies. Methacarn-fixed tissues were stained with anti-proliferating cell nuclear antigen (anti-PCNA) antibody (clone PC10; Novocastra, Newcastle upon Tyne, UK), anti-vimentin antibody (clone V9; Dako, Glostrup, Denmark), and anti-glial fibrillary acidic protein (anti-GFAP) antisera (Dako),
and formalin-fixed tissues were stained with anti-rat monocyte and macrophage antibody (clone ED1; Serotec, Oxford, UK) and anti-rat macrophage antibody (clone ED2; Serotec) by the labeled streptavidin biotin kit (Dako, Carpinteria, CA) as described previously. \(^{18,19}\) Trypsinization (0.1% trypsin from hog pancreas, Nacalai Tesque, Kyoto, Japan) for 15 minutes at 37°C was necessary for ED1 and ED2 visualization. ED1 recognizes macrophages, monocytes, and dendritic cells whereas granulocytes are negative. ED2 recognizes resident macrophages whereas monocytes, dendritic cells, and granulocytes are negative.\(^{20}\)

**Electron Microscopy**

Tissues fixed in 0.5X Karnovsky solution were post-fixed in 2% OsO\(_4\) and processed for Luveak-812 embedding. Sections, 1 \(\mu\)m thick, stained with toluidine blue were used for light microscopy and to locate areas for electron microscopy. Thin sections stained with uranyl acetate and lead citrate were examined with the Hitachi H-600 electron microscope.

**Quantitative Analysis**

For estimation, 4-\(\mu\)m-thick eye sections of 75-mg/kg MNU-treated and control S-D rats that included the ora serrata and the optic nerve obtained from two to four rats killed at the same interval were used. The whole retina thickness was measured from the internal limiting membrane up to the pigment epithelium, and the thickness of each layer was measured. The number of PCNA- and ED1-labeled cells per 0.1-mm and 1-mm length of the section, respectively, in the posterior pole of the retina was counted.

**Results**

All treated and control S-D rats were in good health and no rats were moribund. However, in the 75-mg/kg MNU group, slight body weight loss (<5%) reaching a maximal decrease 2 days after the treatment occurred, but thereafter the rats in this group gained body weight similar to the control rats. No body weight loss was seen in the 50-mg/kg MNU group. Retinal degeneration, which was always bilateral with similar severity, occurred in MNU-treated rats. None of the untreated animals killed at the end of the experiment developed any morphological changes of the retina. Table 1 shows the time-dependent and dose-related retinal degeneration in MNU-treated and untreated S-D rats. Single intraperitoneal doses of 50 mg/kg MNU produced only a small number of retinal lesions. However, 75 mg/kg MNU caused time-dependent retinal degeneration in all treated rats, the severity of which increased with the passage of time. Therefore, the animals treated at 75 mg/kg MNU were studied in more detail and will be described below. In all cases, the posterior portion of the retina around the optic nerve was more severely damaged, which indicates that the damage proceeded from the central to peripheral retina. Therefore, the posterior pole of the retina was recorded.

Light microscopically, compared with the untreated rat retina (Figure 1a), the first evidence of retinopathy 24 hours after dosing was the pyknosis and karyorrhexis of the photoreceptor nuclei and shortening of the inner segment and the disorientation of the outer segment of the photoreceptor layer (Figure 1b). The destruction of the photoreceptor segments and pigment epithelial layer was easily recognized by the PAS-negative inner segment, PAS-positive outer segment, and PAS-negative pigment epithelial cells. The pigment epithelial layer was scarcely seen at this time point. After 48 and 72 hours, the karyorrhexis of the photoreceptor cells...
progressed, and widespread destruction of the photoreceptor segment was seen (Figure 1, c and d). The nuclear destruction was most prominent at 48 hours, and cells with mitotic figures were present in the inner nuclear layer and among these dying cells at 72 hours. The pigment epithelial layer became indistinct. At day 7 (Figure 1e), as the result of photoreceptor cell depletion, active signs of nuclear degeneration in the outer nuclear layer became indistinct, and the PAS-positive outer segment was lost.

Figure 3. TUNEL staining in untreated and 75-mg/kg MNU-treated S-D rats. a: Untreated control. b and c: At 24 and 48 hours after the treatment, respectively. The majority of the photoreceptor cell nuclei are labeled. d: At 72 hours after the treatment. The number of labeled cells has decreased compared with b and c. e: At 7 days after the treatment. No staining is seen. TUNEL method, magnification, ×300.

Figure 4. PCNA-positive cells (arrowheads) in 75-mg/kg MNU-treated S-D rats. a: At 48 hours after the treatment. PCNA-labeled nuclei are seen in the inner nuclear layer. b: At 72 hours after the treatment. Many PCNA-labeled nuclei are seen in the inner nuclear layer and between inner nuclear layer and choroid. PCNA, magnification, ×300.
a lack of an inflammatory response despite the large number of degenerative elements.

Retinal width of specific retinal layers in animals after MNU treatment were quantified sequentially (Figure 2). Compared with the untreated control rats, there was a progressive decrease in width in MNU-treated rats, representing a 33% loss in 7 days and a 58% loss in 35 days. Particularly, a drastic decrease in the outer nuclear layer and photoreceptor segments as well as outer plexiform layer was noticeable, which disappeared 7 days after the treatment. However, the remaining retinal structures, the ganglion cell layer, inner plexiform layer, and inner nuclear layer were well preserved with the degeneration limited to the photoreceptor cells, but the continuous pigment epithelial layer became indistinct.

The TUNEL method did not label retinal cells of untreated rats (Figure 3a). At 24 and 48 hours after dosing (Figure 3, b and c), TUNEL staining revealed nuclear labeling in a great majority of the photoreceptor cells, even in the cells in which the nuclear destruction was not obvious. At 72 hours (Figure 3d), the numbers of labeled cells decreased, and at 7 days (Figure 3e), no staining was observed because most of the photoreceptor cells had disappeared. TUNEL staining was restricted to photoreceptor cells, and other cells were invariably negative. Quantitative analysis at different time points could not be performed due to massive karyolysis, which resulted in difficulty counting the exact number of photoreceptor nuclei. In contrast to these apoptotic cells, PCNA-labeled nuclei located in the inner nuclear layer (Figure 4a) and very rarely in the outer nuclear layer appeared in 48 hours and reached a maximum at 72 hours with the positive nuclei located at the inner nuclear layer and between the inner nuclear layer and choroid (Figure 4b). The positivity drasti-

![Image](image-url)

**Figure 5.** PCNA labeled nuclei per 0.1 mm length of the section in the posterior pole of the retina of 75 mg kg MNU-treated SD rats.

However, at 7 and 21 days after the treatment (Figure 1f), several layers of cells remained between the inner nuclear layer and the choroid. Eventually, 35 days after the treatment, the inner nuclear layer was in direct contact with the choroid or had a single layer of cells between them (Figure 1g). In this late stage (21 and 35 days after the treatment), vacuole formation (cystoid degeneration) was seen in the inner nuclear layer and in the inner plexiform layer, which increased in number with the passage of time. During the degeneration, there was characteristically
cally decreased at day 7. Figure 5 shows the quantitative analysis of PCNA-labeled proliferative cells. In untreated rats, GFAP and vimentin labeled only glia on the inner surface of the retina. However, 48 and 72 hours after the treatment, parallel to the appearance of PCNA-positive cells, radically oriented cell processes corresponding to Müller cells revealed accumulated GFAP and vimentin with a similar distribution (Figure 6, a and b). This GFAP and vimentin expression was maximal at 72 hours after the treatment and became weaker 7 days after the treatment. Macrophages as recognized by ED1 positivity appeared in the choroid 48 hours after the treatment and soon within the retina (Figure 7, a–c). Figure 8 shows the sequential changes of the intraretinal infiltration of the ED1-positive macrophages. Retinal infiltration was maximal 7 and 21 days after the treatment and gradually decreased at day 35. However, ED2 remained negative throughout the experiment.

Electron microscopically, compared with the untreated S-D rats (Figure 9a), at 24 hours after the treatment, almost all of the photoreceptor cells revealed nuclear condensation, leaving Müller cell nuclei intact (Figure 9b), and at 48 hours, karyolysis of the photoreceptor nuclei occurred. At 24 and 48 hours, with the progressive degeneration of the cytoplasmic structure, the inner segment of the photoreceptors was distorted and the outer segment showed disorderly arrangement of the lamellar disks. At 72 hours, numerous phagocytes occurred (Figure 10). Müller cells appeared to be resistant; they increased in number and extended their processes into the spaces previously occupied by the photoreceptor cells. At 7 days after the treatment, Bruch’s membrane and endothelial cells of the choriocapillaries were intact, but 21 days after the treatment, neovascularization consisting of fenestrated vessels was seen between inner nuclear cells and Bruch’s membrane, and cystoid space formation was seen (Figure 11). Finally, 35 days after the treatment, inner nuclear cells were in direct contact with Bruch’s membrane or with pigment epithelium and/or with Müller cells. Compared with the continuously arranged pigment epithelium above Bruch’s membranes seen in the control rats (Figure 12a), already 24 hours after the treatment, the pigment epithelium was not continuously arranged. When present, phagocytosis and vacuolization was seen in the cytoplasm, extending thin cell
Figure 9. Electron micrograph showing photoreceptor nuclei of the untreated and 75-mg/kg MNU-treated S-D rat. a: Untreated control. b: At 24 hours after the treatment. Photoreceptor cells show hyperchromatic nuclei, but Müller cells are unaffected. Magnification, X 4500.

Figure 10. Electron micrograph of the photoreceptor layer 72 hours after 75-mg/kg MNU treatment in the S-D rat. Note the numerous phagocytes. Magnification, X 3000.

Discussion

Despite the numerous studies on the carcinogenicity of MNU, very little is known about the retinal changes. We found that a single intraperitoneal administration of 75 mg/kg MNU induced progressive retinal degeneration in all treated rats. At 50 mg/kg, MNU did not induce retinopathy in all treated rats. Sequential observation of the retinal degeneration induced by MNU revealed restricted involvement of the retinal tissue, with destructive changes selective to the photoreceptor cells. Age-related photoreceptor degeneration occurs in F344 albino rats over 1 year of age whereas S-D rats do not show any obvious change at 1.5 years of age. Albino rats are highly susceptible to phototoxic retinopathy, light levels within the top row of the cages should be <60 lux. However, the light level in the animal room was <60 lux, and the untreated rat retina was free of damage. MNU is a direct acting alkylating agent and has a half-life of <1 hour under physiological conditions. DNA adduct formation was seen in MNU-treated rats restricted to the photoreceptor nuclei, suggesting that DNA alkylation is a direct toxic effect of MNU. Thus, the retinal degeneration observed in

processes along Bruch's membrane where neighboring pigment epithelium was supposed to be present (Figure 12b).

The severity of retinal lesions in B-N rats 7 days after the treatment with either 50 or 75 mg/kg MNU was comparable to that in S-D rats. Again, the 50-mg/kg dose was less effective. The advantage of using colored rats is that the movement of the pigment epithelium is easily seen. Accordingly, in the 75-mg/kg group, the pigment epithelium was discontinuous above Bruch's membrane, and migration of the swollen pigment epithelium was detected between the inner nuclear layer and choroid and within the inner nuclear layer (Figure 13).

The pathological changes due to MNU were restricted to the retina in S-D and B-N rats. The optic nerve was normal, and the anterior ocular structures including the lens showed no apparent abnormality. All other organs and tissues of the MNU-treated animals were normal. Therefore, retinal degeneration was the only MNU-related pathological change during the observation period.
the present study is due to direct DNA damage caused by MNU and not by phototoxicity or age-related phenomenon.

Apoptosis occurs during normal retinal development.\textsuperscript{23} In mutant mice and rats with retinal degeneration, apoptosis is the common pathway of photoreceptor cell death.\textsuperscript{12-16} Chemicals usually cause cell damage due to necrosis. However, because internucleosomal DNA fragmentation is the hallmark of apoptosis, our findings have demonstrated that MNU-induced retinal degeneration was due to apoptosis. By the TUNEL method, the positively stained cells were restricted to photoreceptor cells, which showed pyknosis and karyorrhexis without the involvement of an inflammatory reaction. Electron microscopically, in agreement with studies of the inherited retinal degeneration mouse,\textsuperscript{12} hyperchromatic nuclei were seen, but the morphological features did not entirely fit the criteria defined by Kerr and coworkers, such as nuclear chromatin condensation at the periphery.\textsuperscript{24,25} A distinct morphological difference may exist between well characterized thymocyte apoptosis and photoreceptor cell apoptosis.

In the inherited retinal degeneration rd and rds mouse, arrested outer segment development followed by photoreceptor cell loss is the primary event, and pigment epithelium appears not to be the site of dysfunction.\textsuperscript{26,27} By contrast, in the RCS rat with the rdy mutation, defect of the phagocytic activity in the pigment epithelium is the primary site of the genetic mutation, which leads to the accumulation of outer segment debris between the neural retina and the pigment epithelium, leading to photoreceptor cell death.\textsuperscript{28,29} Various chemicals produce retinotoxicity in laboratory animals, which results in selective destruction of the photoreceptor cells.\textsuperscript{30-33} Among the chemicals, urethane causes photoreceptor degeneration without initial damage to the pigment epithelium.\textsuperscript{34,35} In contrast, pigment epithelial necrosis is the primary site of toxicity with subsequent photoreceptor outer segment disruption by 1,4-bis(4-aminophenoxy)-2-phenylbenzene (2-phenyl-APB-144).\textsuperscript{33} Although the final event is the photoreceptor cell degeneration, the primary target differs with each model. In the present model, the primary target was the photoreceptor cell death due to apoptosis. In agreement with the MNU-induced hamster model,\textsuperscript{8} the observation on colored B-N rats disclosed that pigment epithelial cells were partially released toward the retinal layer in parallel to photoreceptor cell degeneration, resulting in the discontinuous lining of the pigment epithelial layer. Therefore, photo-
receptor cell degeneration followed by pigment epithelial cell migration resembles retinitis pigmentosa in humans. 64

Müller cells normally do not express GFAP; they accumulate GFAP with respect to their reaction to injury and degeneration. 37-39 Thus, GFAP accumulation seems to be a generalized response to several types of injuries. Moreover, Müller cells are positive with vimentin indicating that vimentin and GFAP coexist within individual fibers. 40 By PCNA labeling, we found proliferation of Müller cells expressing GFAP and vimentin in parallel to photoreceptor cell degeneration. Müller cells occupied the space where the photoreceptor cells were present. Thus, the Müller cell phenomenon may be a response to stabilize and to preserve the damaged retina. Macrophage infiltration has been observed during retinal degeneration in mutants. In the present model, soon after the Müller cell proliferation, macrophage infiltration was seen in the degenerative retina. Electron microscopically, in agreement with the urethane-induced retinopathy, incorporation of choroidal phenotype (fenestrated) retinal vessels was seen in the retina. Macrophage infiltration into the retina may occur via fenestrated vessels or via direct transmission through Bruch’s membrane. The ED1-positive but ED2-negative macrophage suggests that it is not a resident macrophage. Marked phagocytosis was observed 72 hours after the MNU treatment. Therefore, Müller cells and macrophages as well as migrated pigment epithelium appear to be responsible for the cell debris removal. In the late stage, although the pathogenesis of cystoid degeneration remains obscure, cystoid formation was found in the inner nuclear layer and inner plexiform layer.

In conclusion, the present findings indicate that MNU had a toxic effect on the eyes, acting directly and selectively on the photoreceptor cells and leading to apoptosis. A secondary reaction was the proliferation of the Müller cells, migration of the pigment epithelium, and infiltration of the macrophages for the cell debris removal. Finally, inner nuclear cells were in direct contact with Bruch’s membrane or with pigment epithelium and/or with Müller cells. At this terminal stage, cystoid degeneration was seen. A better understanding of the mechanism of photoreceptor cell death by MNU, an environmental toxin, will provide an additional model for retinitis pigmentosa in humans.

Acknowledgments

The authors thank Ms. T. Akamatsu for technical assistance and Ms. M. Fukuchi for preparing the manuscript.

References