Review

N-Methyl-*N*-nitrosourea-induced Retinal Degeneration in Animals

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Retinitis pigmentosa (RP) is a human disease characterized by loss of photoreceptor cells leading to visual disturbance and eventually to blindness. Establishment of reliable animal models is essential for better understanding of this disease and approaches to therapeutic intervention. *N*-Methyl-*N*-nitrosourea (MNU)-induced retinal degeneration is highly reproducible, and involves photo-

receptor cell loss that ends approximately 7 days after a single systemic administration of MNU. Although the triggering mechanisms of pathogenesis are different and the apoptosis cascade may differ from human RP, MNU-induced photoreceptor cell loss is due to apoptosis. Here, we describe disease progression and therapeutic trials of MNU-induced retinal degeneration in animals.

Key words: retinitis pigmentosa, retinal degeneration, photoreceptor cell, apoptosis, *N*-methyl-*N*-nitrosourea

I. Introduction

Retinitis pigmentosa (RP) is a well-known clinical entity that was defined by Donders more than a century ago [12]. It comprises a heterogeneous group of inherited human disorders that involve primary degeneration of photoreceptor cells following detachment of retinal pigment epithelial cells from Bruch's membrane that accumulate around the retinal blood vessels, resulting in clinically observed bone-spicule deposition [35, 56]. This category of disease was assigned the term RP because it was initially thought to be a result of an inflammatory process. The incidence of RP is approximately 1/4000, making it one of the major causes of blindness in humans [2]. Although several genes encoding photoreceptor-specific proteins have been implicated in RP, the final common pathway of the disease is apoptotic cell death of photoreceptor cells [52]. There are several animal models for RP in which animals that carry mutant genes develop retinal degeneration [8, 39, 53]. The mammalian eye is highly sensitive to toxic substances, and certain chemicals have been shown to cause retinal degeneration

in animals [19, 25, 34]. Herrold [18] was the first to report photoreceptor cell degeneration in Syrian golden hamsters after systemic administration of *N*-methyl-*N*-nitrosourea (MNU), an alkylating agent. Although the triggering mechanisms of pathogenesis are different from human RP, our recent findings indicate that MNU-induced photoreceptor cell degeneration is due to apoptosis, and that it may be useful for testing of therapeutic strategies.

II. MNU-induced Retinal Degeneration in Different Animal Species

We examined the retinal response of Sprague-Dawley rats and Lewis rats at different ages to various doses of MNU [42]. Eye sections cut at the central part of the eyeball, parallel to the optic axis and nerve (including the ora serrata and optic nerve), were used for evaluation. In both rat strains, regardless of gender, rats developed retinal degeneration characterized by selective photoreceptor cell loss over a 7-day course after intraperitoneal (ip) administration of MNU at a dose of >90, >50 and ≥ 35 mg/kg body weight at 21, 50 and 150 days of age, respectively. Susceptibility of photoreceptor cells to development of MNU-induced retinal degeneration increases with increasing age. When

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Table 1. Characteristics of MNU-induced retinal degeneration in different animal species

Species	Age	MNU (mg/kg)	Route of administration	Site of origin	Fate of retinal pigment epithelial cells
Mouse	7 weeks	60	ip	Posterior pole	No migration
Rat	50 days	60	ip	Posterior pole	Intraretinal migration without contact with blood vessels
Hamster	50 days	90	ip	Posterior pole	Contact with intraretinal blood vessels
Shrew	7 weeks	65	ip	Posterior pole	No migration
Monkey	Young adult	40	iv	Equatorial zone	No migration

ip, intraperitoneally; iv, intravenous

≥ 50 mg/kg MNU was administered to newborn rats (day 0), they developed retinal dysplasia characterized by rosette formation. Retinal dysplasia develops when 60 mg/kg MNU is administered to 0- or 3-day-old C57BL mice, whereas retinal degeneration occurs when this dose is administered after 11 days of age [41]. From the ontogenetic point of view, retinal maturation is apparently essential for photoreceptor cell loss. We showed that retinal degeneration can be induced by a single systemic administration of MNU in a variety of adult animals, including rodents (mouse, rat and hamster) [37, 38, 40, 48, 60], insectivora (shrew; *Suncus murinus*) [51], and non-human primates (monkey; *Macaca fuscata*) [51]. Thus, MNU-induced photoreceptor cell loss is apparently a phylogenetically universal phenomenon. However, the dose of MNU necessary for induction of photoreceptor cell loss over a 7-day course differs among species (Table 1). In human RP, photoreceptor cell apoptosis starts in the equatorial zone of the retina and spreads centrally and peripherally [56]. In monkeys, MNU-induced photoreceptor cell injury also begins in the equatorial zone, whereas, in other species, the lesions originate in the posterior pole. Species differences in disease progression may reflect differences in rod and cone cell distribution in the retina. In human RP, after the photoreceptor cell loss, migrating pigment epithelial cells surround intraretinal blood vessels. Intraretinal migration of retinal pigment epithelial cells is seen in both rats and hamsters, but contact between migrating pigment epithelial cells and blood vessels has only been observed in hamsters. Migration of pigment epithelial cells has not been observed in mice, shrews or monkeys. Thus, although photoreceptor cell loss is the common primary event, the fate of retinal pigment epithelial cells differs among species.

III. Time Course of MNU-induced Retinal Degeneration in Rats

In our study in which 50-day-old Sprague-Dawley albino and Brown-Norway non-albino rats received a single ip injection of 75 mg/kg MNU, all treated rats developed time-dependent retinal degeneration [37, 38]. In all treated rats, the posterior pole around the optic nerve was the area most severely damaged, indicating that the damage proceeded from the central to the peripheral retina. At 24 hr after MNU administration, pyknosis and karyorrhexis of the

photoreceptor cell nuclei and shortening and disorientation of photoreceptor segments were observed; nuclear destruction was most prominent at 48 hr. Loss of photoreceptor nuclei progressed, and mitotic figures were present in the inner nuclear layer and in dying photoreceptor cells at 72 hr (Fig. 1b). At day 7, as a result of photoreceptor cell loss, active signs of nuclear degeneration in the outer nuclear layer became indistinct (Fig. 1c), and photoreceptor segments were lost. Finally, at 35 days after MNU treatment, vacuole formation (cystoid degeneration) was seen in the inner and outer retina, and the inner nuclear layer was either in direct contact with the choroid or was separated from it by a single layer of cells (Fig. 1d). In this model, photoreceptor cells were evenly damaged. In rats, although sparse, randomly distributed cones are present, most of the photoreceptor cells are rods responsible for scotopic vision [27].

Alkylating agents are cytotoxic, and produce methylated DNA adducts in target cells [43, 44]. Amex-fixed, paraffin-embedded sections were processed for immunohistochemical detection of methylated DNA adducts. The immunohistochemical method and the characteristics of the antibody used have been reported previously [57]. High levels of DNA adducts (7-methyldeoxyguanosine) have been detected in photoreceptor cell nuclei 12 and 24 hr after MNU exposure (Fig. 2). Cells exposed to an alkylating agent undergo apoptotic cell death when DNA damage is not repaired [50]. Internucleosomal DNA fragmentation is seen in the retina 12 hr after MNU exposure [42]. At 24 hr after MNU treatment, almost all treated photoreceptor cell nuclei exhibit nuclear condensation, and Müller cell nuclei are intact, as shown by the electron microscopic images in Fig. 3b. Also, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling (TUNEL) signals appear selectively in photoreceptor cell nuclei, and peak 48 hr after MNU treatment (Fig. 4). These findings all suggest that death of photoreceptor cells after MNU treatment is due to an apoptotic mechanism. However, electron microscopically, in agreement with inherited retinal degeneration mice [9], photoreceptor cells showed hyperchromatic nuclei, but the morphological features did not entirely fit the criteria defined by Kerr and coworkers, such as nuclear chromatin condensation at the periphery [55]. Moreover, TUNEL signals are sporadically seen in inherited retinal degeneration mice [9], TUNEL signals were diffusely seen after MNU. Compared with mice that

Fig. 1. Retina of Sprague-Dawley rats: untreated controls and MNU-treated rats (75 mg/kg). **a:** Untreated control. **b, c, and d:** 72 hr, and 7 and 35 days after MNU treatment. **b:** Destruction of photoreceptor cells is shown, and cells with mitotic figures are present in the inner nuclear layer and among the dying cells. **c:** Due to photoreceptor cell depletion, active signs of photoreceptor cell degeneration are indistinct. **d:** Inner nuclear layer cells are in direct contact with choroids, or are separated from them by several layer of cells. Note vacuole formation (cystoid degeneration) in the inner nuclear layer and in the inner plexiform layer. 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.

Fig. 2. 7-Methyldeoxyguanosine (7-medGua) DNA adducts in MNU-treated (75 mg/kg) Sprague-Dawley rat 24 hr after treatment. 7-medGua-positive signals were observed in almost all photoreceptor cell nuclei located at ONL. Abbreviations: refer to Fig. 1.

carry mutant genes, MNU might have caused different degrees of injury to photoreceptor cells. Apoptosis is regulated by a complex mechanism, and the Bcl-2 family of proteins includes important regulators of apoptosis. This family includes Bcl-2, which prevents apoptosis, and Bax, which can induce cell death [14]. Compared with MNU-untreated retinas, Bcl-2 expression decreased and Bax

Fig. 3. Electron micrograph showing photoreceptor nuclei of untreated and MNU-treated (75 mg/kg) Sprague-Dawley rats. **a:** Untreated control. **b:** 24 hr after MNU treatment. Photoreceptor cells have hyperchromatic nuclei, but Müller cells are unaffected. Bar=3.5 μ m.

expression increased 12 and 24 hr after MNU treatment, respectively, as examined by Western blotting [57]. Cas-

Fig. 4. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick-end labeling (TUNEL) staining in MNU-treated (75 mg/kg) Sprague-Dawley rats 48 hr after MNU treatment. Majority of photoreceptor cell nuclei located at ONL are selectively labeled. Abbreviations: refer to Fig. 1.

pases, which are members of the cysteine protease family, apparently play a decisive role as mediators of neuronal or retinal apoptosis, or both [7, 17, 23, 28, 31]. Caspase-3, -6 and -8 activities peak 72 hr after MNU treatment [57]. Thus, the MNU-induced photoreceptor cell apoptosis cascade involves up-regulation of Bax protein, down-modulation of Bcl-2 protein, and activation of caspase family proteins.

In parallel to photoreceptor cell loss, proliferating cell nuclear antigen (PCNA)-labeled nuclei appear in the inner nuclear layer and between the inner nuclear layer and choroid 48 hours after MNU treatment, and reach maximum levels at 72 hr (Fig. 5a). These PCNA-positive cells possess radically oriented vimentin-positive cell processes, corresponding to Müller cells (Fig. 5b). As a result of photoreceptor cell loss, proliferation of Müller cells occurs. As the Müller cells proliferate, they extend their cell processes and occupy the space previously occupied by photoreceptor cells. Thus, Müller cells may be responsible for the stabilization and preservation of the damaged retina. At 72 hr after MNU treatment, marked phagocytosis is seen in these proliferating Müller cells, and, at 7 and 21 days after MNU treatment, ED1-positive macrophage infiltration appears within degenerative retina. The fate of retinal pigment epithelial cells has been followed in Brown-Norway non-albino rats [37]. At 72 hr after MNU treatment, detachment of the pigment epithelial cells from Bruch's membrane was seen, and, at day 7, 21 and 35, migrating pigment-containing cells were present in all layers of the retina. In agreement to inherited

Fig. 5. Proliferating cell nuclear antigen (PCNA) and vimentin staining in retina of MNU-treated (75 mg/kg) Brown-Norway rats 72 hr after MNU treatment. **a:** PCNA staining. **b:** vimentin staining. Proliferating Müller cell processes can be seen.

retinal degeneration mice [5, 47], Müller cells, macrophages and migrated pigment epithelial cells are involved in removal of apoptotic cells. However, the migrated melanin-containing cells were not in contact with blood vessels. Therefore, the primary event was the destruction of photoreceptor cells via an apoptotic mechanism, followed by Müller cell proliferation, macrophage infiltration and retinal pigment epithelial cell migration (all of which are involved in cell debris phagocytosis). The result was a thin remnant of the retina that lacked photoreceptor cells.

IV. Disease Control of MNU-induced Retinal Degeneration

The rescue of retinal neural cell loss has been studied in several animal models. Gene therapy targeting apoptosis-related genes such as the Bcl-2 [11], c-fos [54] and ribozyme [15] genes is effective in reducing photoreceptor cell death. It is theoretically possible to suppress the photoreceptor cell apoptosis by inhibiting the apoptosis cascade. Several inhibitors of caspase, such as YVAD-CMK, YVAD-CHO [21, 30, 31], IETD-FMK [49], DEVD-CHO and DEVD-FMK [10, 21–23, 31], have been applied in retinal degeneration models. MNU-induced photoreceptor cell apoptosis involves Bcl-2 family proteins and activation of caspase-3, -6 and -8 [57]. In our study, Ac-DEVD-CHO, a caspase-3 inhibitor, was injected intravitreally at a dose of 4000 ng, twice at 0 and 10 hr after 60 mg/kg MNU was administered to 50-day-old rats [58]. This Ac-DEVD-CHO injection significantly reduced the TUNEL index 24 hr post-MNU in the central retina (83.7% vs. 71.8%) and peripheral retina (79.5% vs. 59.7%), and selectively rescued photoreceptor cell loss 7 days after MNU administration. Photoreceptor cell ratio [(thickness of the outer nuclear layer and photoreceptor layer/total retinal thickness)×100] was 3.3% in the central retina and 6.0% in the peripheral retina at 7 days

Fig. 6. Effect of Ac-VEVD-CHO on photoreceptor ratio in untreated control and MNU-treated Sprague-Dawley rats. Photoreceptor ratio: (photoreceptor cell thickness/total retinal thickness) $\times 100$. This ratio was evaluated in the central retina (average of approximately 400 μm from both sides of the optic nerve) and peripheral retina (average of approximately 400 μm from both sides of the ciliary body). Each data value is the mean of 10 rats $\pm$ SE. * $p < 0.05$ and ** $p < 0.01$.

Fig. 7. Comparison of retinal damage ratio after Ac-VEVD-CHO administration in MNU-treated Sprague-Dawley rats. Retinal damage ratio: (length of damaged retina with less than 4 rows of photoreceptor cells in the outer nuclear layer/whole retinal length) $\times 100$. ** $p < 0.01$.

after MNU treatment without Ac-DEVD-CHO. In the rats injected with Ac-DEVD-CHO, photoreceptor cell ratio was significantly preserved: 11.3% in the central retina and 42.5% in the peripheral retina at 7 days after MNU treatment (Fig. 6). Without Ac-DEVD-CHO, the retinal damage ratio [(retinal length where photoreceptor cell nuclei across the width of the retina comprise less than 4 rows/whole retinal length) $\times 100$] at 7 days after MNU was 98.5%, whereas Ac-DEVD-CHO significantly reduced this value to 54.4% (Fig.

7). Caspase-3 inhibitor strongly suppressed MNU-induced photoreceptor cell apoptosis and significantly preserved photoreceptor cells in the peripheral retina. In MNU-induced retinal injury in rats, the damage proceeds from the central to peripheral retina, and the central retina is more severely damaged. Ac-DEVD-CHO suppressed and/or delayed the progression of photoreceptor cell damage. However, the relatively rapid and extensive cell loss in the central retina was beyond the rescue capacity of Ac-DEVD-CHO. Ac-DEVD-CHO also delays disease progression in *rd* gene-carrying mice, which normally develop retinal degeneration early in life [59].

We examined the effects of nicotinamide (NAM), a water-soluble B-group vitamin, on MNU-induced retinal degeneration [24]. DNA damage induced by alkylating agents activates poly (ADP-ribose) polymerase (PARP), which repairs injured DNA using nicotinamide adenine dinucleotide (NAD^+) as a substrate [6]. This results in the depletion of NAD^+ pools [1] and consequent ATP deficiency, leading to energy loss [4, 61]. ATP depletion is believed to be one of the most critical factors leading to cell death [6]. Exogenously applied NAM, a precursor of NAD^+ , may counteract the depletion of NAD^+ . NAM also acts as a PARP inhibitor [16]. Another PARP inhibitor, 3-aminobenzamide, which is not a NAD^+ precursor, counteracts PARP activation and prevents depletion of NAD^+ [13], and has been found to ameliorate *N*-methyl-D-aspartate-induced apoptosis in the rat inner retina [29, 30]. NAM acts as a vasodilator [20], and may mitigate hypoxia of the outer retina. NAM suppresses MNU-induced photoreceptor cell loss in a dose-dependent manner when injected subcutaneously immediately after ip injection of 60 mg/kg MNU. In rats, a dose of ≥ 25 mg/kg NAM completely suppresses photoreceptor cell loss, and

Fig. 8. Dose-response effects of nicotinamide (NAM) administered immediately after MNU on retinal damage evaluated by photoreceptor ratio at the posterior pole in Sprague-Dawley rats 7 days after MNU treatment. Photoreceptor ratio: (photoreceptor cell thickness/total retinal thickness)×100. ** $p < 0.01$. Each group consists of 10 eyes.

Fig. 9. Time-response effects of 1000 mg/kg nicotinamide (NAM) on retinal damage evaluated by photoreceptor ratio at the posterior pole in Sprague-Dawley rats 7 days after MNU injection. ** $p < 0.01$. Each group consists of 6 to 10 eyes.

10 mg/kg partially suppresses photoreceptor cell loss (Fig. 8). In addition to morphology, both scotopic and photic electroretinographic (ERG) recordings have shown that both rod and cone photoreceptor cells are functionally well protected from MNU damage by 1000 mg/kg NAM [26]. Also, 1000 mg/kg NAM completely suppresses photoreceptor cell loss when administered up to 4 hr after MNU, and partially suppresses photoreceptor cell loss when administered 6 hr after MNU treatment; NAM is ineffective when administered 12 hr after MNU (Fig. 9). Levels of DNA adducts (7-medGua) in the retina are not decreased in animals treated with both MNU and NAM. Thus, NAM does not prevent DNA damage, but seems to repair damaged DNA.

In human infants, it has been demonstrated that docosa-

Fig. 10. Effects of different semipurified 10% fatty acid diets on retinal damage ratio in MNU-treated Sprague-Dawley rats. LA, linoleic acid; PA, palmitic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

hexaenoic acid (DHA) is important for maturation of photoreceptor cells [3]. In a study in which DHA, palmitic acid (PA), oleic acid (OA) or arachidonic acid (AA) was added to retinal neurons in culture, DHA supplementation decreased photoreceptor cell death, whereas PA, OA and AA did not decrease photoreceptor cell degeneration [45, 46]. In contrast to this beneficial effect of DHA supplementation, AA (a member of the n-6 PUFA family) has harmful and proapoptotic effects in some nerve tissues [33]. A diet rich in DHA may enhance retinal function and influence photoreceptor cell survival. In our study, 50-day-old Sprague-Dawley rats were given 50 mg/kg MNU, and were then switched to 1 of 5 different diets containing the following fatty acids at the following weight percentages: 10% linoleic acid (LA); 9.5% PA and 0.5% LA; 9.5% eicosapentaenoic acid (EPA) and 0.5% LA; 4.75% EPA plus 4.75% DHA and 0.5% LA; 9.5% DHA and 0.5% LA [36]. Retinal samples were obtained 20 weeks after MNU injection or when rats developed MNU-induced mammary tumors with a diameter of ≥ 1 cm. In rats, a single ip dose of 60 to 75 mg/kg MNU induces retinal degeneration and photoreceptor cell loss over a 7-day course; 50 mg/kg MNU is less toxic [38]. The available evidence suggests that a dose of 50 mg/kg MNU will allow assessment of changes in incidence and severity of retinal damage in rats fed different fatty acid diets. There was no significant difference in the mean period between MNU injection and eye sampling among the 5 diet groups. The incidence of retinal degeneration was highest in the LA group (88%, 15/17), followed by EPA (73%, 11/15), EPA-plus-DHA (53%, 9/17), PA (41%, 7/17) and DHA (0%, 0/15). Severity of retinal degeneration (retinal damage ratio) showed a similar trend across diet groups (Fig. 10). MNU-induced retinal degeneration was prevented in rats fed the diet containing 9.5% DHA (4.75% DHA was less effective), whereas it was accelerated in rats fed the 10% LA diet. However, conjugated DHA, a group of positional and geometric isomers of DHA, was ineffective (unpublished observation).

V. Concluding Remarks

MNU, a potent alkylating agent, induces photoreceptor cell loss in a variety of animal species. This photoreceptor cell loss is attributed to restriction of DNA adduct formation in photoreceptor cell nuclei, leading to apoptosis accompanied by up-regulation of Bax protein, down-modulation of Bcl-2 protein, and activation of caspase family proteins. Many novel therapeutic approaches to control of photoreceptor cell death have been evaluated. The first strategy we investigated for therapy of MNU-induced retinal degeneration was shutting down the apoptosis cascade; we tested a caspase-3 inhibitor, which partially suppressed disease progression. Next, we tested the ability of NAM to suppress photoreceptor cell apoptosis via repair of damaged DNA or other mechanisms, and found it to be relatively effective. Also, we found that dietary DHA supplementation counteracts MNU-induced photoreceptor cell loss. Using MNU-induced retinal degeneration in animals, studies are ongoing to elucidate the mechanisms suppressing photoreceptor cell apoptosis.

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