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Review

Animal models for retinitis pigmentosa induced by MNU; disease progression, mechanisms and therapeutic trials

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Summary. Retinitis pigmentosa (RP) is a group of inherited neurodegenerative diseases in humans characterized by loss of photoreceptor cells leading to visual disturbance and eventually to blindness. A single systemic administration of *N*-methyl-*N*-nitrosourea (MNU) causes retinal degeneration in various animal species. The retinal degeneration is highly reproducible, and the photoreceptor cell loss occurs within seven days after MNU administration via apoptosis resembling human RP. Here, we describe the disease progression, disease mechanisms, and therapeutic trials of MNU-induced retinal degeneration.

Key words: Retinitis pigmentosa, *N*-methyl-*N*-nitrosourea (MNU), Apoptosis, Poly (ADP-ribose) polymerase (PARP), Caspase

Introduction

Retinitis pigmentosa (RP) is a well-known clinical entity that was named by German physician Franz Donders in 1857. It is characterized by early nyctalopia and non-inflammatory, bilateral, progressive, degenerative pigmentary retinopathy. The loss of photoreceptors, first rods and then cones, is followed by perivascular pigment deposition within the retina (Hartong et al., 2006). Thus, the word "retinitis" is a misnomer, because retinal inflammation does not play a major role. RP is a heterogeneous group of inherited retinal disorders. More than 160 different mutations in genes coding for proteins with remarkably diverse functions result in rod photoreceptor degeneration (www.sph.uth.tmc.edu/retnet). The fundus typically shows intraretinal pigmentation, referred to as bone-spicule deposits, created by the migration of retinal pigment epithelial (RPE) cells and their deposition around retinal vessels. There is currently no cure or effective therapy for the treatment of RP.

Animal models of retinal degeneration are important for a better understanding of human RP, particularly in light of the search for treatments (Dalke and Graw, 2005). Mice carrying the *rd* (rodless retina or retinal degeneration) gene (now *Pde6b*), a defect in the ß subunit of cyclic guanosine monophosphate phosphodiesterase (cGMP PDE), develop retinal degeneration early in life. Retinal development in *rd* mice is comparable to that in normal mice at 8 days of age; however, a reduction in the number of photoreceptor cells becomes apparent at 11 days of age, and photoreceptor cells are completely missing or reduced to a single layer of cells by 20 days of age (Nambu et al., 1996). In *rds* (retinal degeneration slow) mice carrying a mutation in the *peripherin/rds* gene, (now *Prph2*), photoreceptor cell loss starts at 2 weeks of age and progresses slowly with complete loss occurring 1 year after birth (Sancho-Pelluz et al., 2008). The RCS (Royal College of Surgeons) rat has a deletion in the gene encoding receptor tyrosine kinase (*Mertk*) expressed in RPE cells, and the inability to phagocytize rod outer segment debris leads to photoreceptor degeneration by apoptosis (Perche et al., 2008); this degeneration begins at 20 days of age, and there are almost no detectable photoreceptors by 60 days of age. The *rd*, *rds*, and *RCS* animals have heterogeneous genetic defects that lead to photoreceptor apoptosis, which is characterized by internucleosomal cleavage and fragmentation (Chang et al., 1993; Tso et al., 1994). Importantly, mutations in these genes have been detected in patients with RP (Perche et al., 2008; Sancho-Pelluz et al., 2008). Moreover, other natural and transgenic animal models of RP are available and have been used to

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understand the mechanisms of the disease, and for the development of therapeutic strategies (Rivas and Vecino, 2009). However, the establishment of additional animal models resembling human RP is desired for a better understanding of disease progression, the analysis of disease mechanisms, and the development of treatments.

Several chemically induced animal models develop retinal degeneration (Voaden, 1991). The mammalian eyes are highly sensitive to toxic substances, and N-methyl-N-nitrosourea (MNU), an alkylating agent that targets photoreceptor cells, may be a good candidate for the induction of retinal damage.

Retinal damage caused by MNU

Induction of retinal degeneration in animals

Herrold (1967) first described retinal degeneration after the systemic administration of MNU to Syrian golden hamsters. Later, apoptosis commitment was reported in MNU-induced retinal degeneration in rats (Nakajima et al., 1996b) and other species (Yuge et al., 1996; Taomoto et al., 1998). A single systemic administration of MNU induces retinal degeneration in both female and male animals, including Insectivora (shrew; *Suncus murinus*), Rodentia (mice of the BALB/c, GRS/A and C57BL strains; rats of the Sprague-Dawley, Lewis, and Brown-Norway strains; and the *Syrian golden hamster*), Lagomorpha (rabbit; Japanese white rabbit), Carnivora (cat), and non-human primates (monkey; *Macaca fuscata*) (Schaller et al., 1981; Ogino et al., 1993; Tsubura et al., 1998). Thus, phylogenetically, MNU-induced retinal degeneration is a universal phenomenon. However, the dose of MNU necessary for the induction of retinal degeneration (photoreceptor cell loss) in a 7-day period differs among species (Table 1). Similar to human RP, the lesion originates from the equatorial zone in monkeys, whereas the lesion originates from the posterior pole in shrews, mice, rats, and hamsters (Tsubura et al., 1998). This difference may be due to relative number and distribution of rods and cones among different animal species. In MNU-treated mice, intravitreal injection of fluorochrome-conjugated peanut agglutinin (PNA) selectively labels cones, and the progressive disappearance of PNA-labeled cones from the posterior to the periphery, in parallel with the rod disappearance, can be seen (Krishnamoorthy et al., 2008). Cone disappearance secondary to rod cell loss occurs in human RP. In rats, retinal degeneration in a 7-day course occurs when >90 mg/kg, >50 mg/kg and ≥35 mg/kg MNU is given to 21-, 50-, and 150-day-old animals, respectively (Nambu et al., 1998b). Ontogenetically, the sensitivity of photoreceptor cells in the development of retinal degeneration induced by MNU increases in parallel with aging. This increased sensitivity may be caused by the accumulation of DNA damage in aged animals. However, the loss of photoreceptor cells is a selective phenomenon in that no other neural cells in the retina were affected by MNU. In contrast to the retinal degeneration process in adult animals, newborn animals treated with MNU exhibit retinal dysplasia characterized by the progressive disorganization of neuroblasts, which leads to rosette formation in the outer neuroblastic/nuclear layer (Nambu et al., 1998a,b).

Migration of RPE cells after photoreceptor cell loss

Fundus pictures of RP patients show characteristic perivascular RPE cell deposition in a bone-spicule configuration. RPE cells in contact with retinal blood vessels are a distinctive feature of human RP. MNU-induced photoreceptor cell loss is followed by intraretinal migration of melanin-containing RPE cells within the retinas of rats and hamsters (Table 1), but RPE cells in contact with blood vessels occur only in hamsters (Taomoto et al., 1998). In rats sacrificed 150 days after MNU administration, RPE cells were scattered within the retina but were not in contact with blood vessels (Nakajima et al., 1996a,b). Migration of RPE cells is not found in shrews, mice, or monkeys. Although photoreceptor cell loss is the primary event in RP and is a common phenomenon in animals, the fate of RPE cells differs among species.

Table 1. Retinal degeneration induced by MNU in different animal species.

Species	Age or Body weight	MNU (mg/kg)	Route of administration	Site of origin	Migration of retinal pigment epithelial cells	Reference
Shrew	7 weeks	65	ip	Posterior pole	No migration	Tsubura et al., 1998
Mouse	7 weeks	60	ip	Posterior pole	No migration	Yuge et al., 1996
Rat	7 weeks	60	ip	Posterior pole	Intraretinal migration without contact with blood vessels	Nakajima et al., 1996b; Yoshizawa et al., 2000
Hamster	7 weeks	90	ip	Posterior pole	Contact with intraretinal blood vessels	Tamoto et al., 1998
Rabbit	2.3-2.5 kg	40	iv	ND	ND	Ogino et al., 1993
Cat	5-10 months	15	iv	ND	ND	Schaller et al., 1981
Monkey	Young adult	40	iv	Equatorial zone	No migration	Tsubura et al., 1998

ip: intraperitoneal; iv: intravenous; ND: not described.

Time-course progression of MNU-induced photoreceptor cell loss

Figure 1 illustrates the time-course progression of retinal degeneration in rats caused by MNU administration. In both colored and albino rats, intraperitoneal administration of 60 or 75 mg/kg MNU causes time-dependent retinal damage in all treated animals (Nakajima et al., 1996a,b; Yoshizawa et al., 1999, 2000). At 24 and 48 h after MNU administration, photoreceptor cells show pyknosis and karyorrhexis of the nuclei and shortening and disorientation of the inner and outer segments. At 72 h, the photoreceptor cell destruction has progressed. At day 7, active signs of photoreceptor degeneration are indistinct due to photoreceptor cell loss, and the inner nuclear layer is either in direct contact with the choroid or is separated from it by a few layers of cells. At this time point, scotopic and photopic electroretinogram (ERG) responses are undetectable, suggesting functional loss of the retina (Fig. 2) (Kiuchi et al., 2003). Internucleosomal DNA fragmentation is seen in the retina 12 and 24 h after MNU administration; the fragmentation peaks 3 days after MNU administration and is almost negligible at day 7 (Fig. 3a). When dying photoreceptor cells are selectively labeled with terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-deoxygenin nick-end labeling (TUNEL) (Fig. 3b) and ultrastructurally compared with untreated retina, the dying photoreceptor cell nuclei appear hyperchromatic (Fig. 3c), and among the dying photoreceptor cells, scattered cells with normal nuclei, presumed to be Müller cells, are seen. These findings suggest that an apoptotic mechanism is responsible for photoreceptor cell death.

Secondary response to photoreceptor cell loss

During photoreceptor apoptosis, mitotic cells appear in the inner nuclear layer and within dying photoreceptor cells 72 h after MNU administration (see Fig. 1). Cells not undergoing apoptosis contain proliferating cell nuclear antigen (PCNA) in their nucleus and vimentin and/or glially fibrillary acidic protein (GFAP) in their proliferating cell processes; these characteristics are indicative of Müller cells. These cells appear 48 h after MNU administration and peak at 72 h. Müller cell proliferation and the extension of their processes within the damaged area may stabilize and preserve the damaged retina. At days 7 and 21 after MNU administration, there is an influx of ED1-positive but ED2-negative bone marrow-derived microglia/macrophages within the retina (Nakajima et al., 1996a,b; Kaneko et al., 2008). Müller cells, macrophages, and migrated RPE cells are involved in the removal of apoptotic cells. By day 21, vacuole formation (cystoid

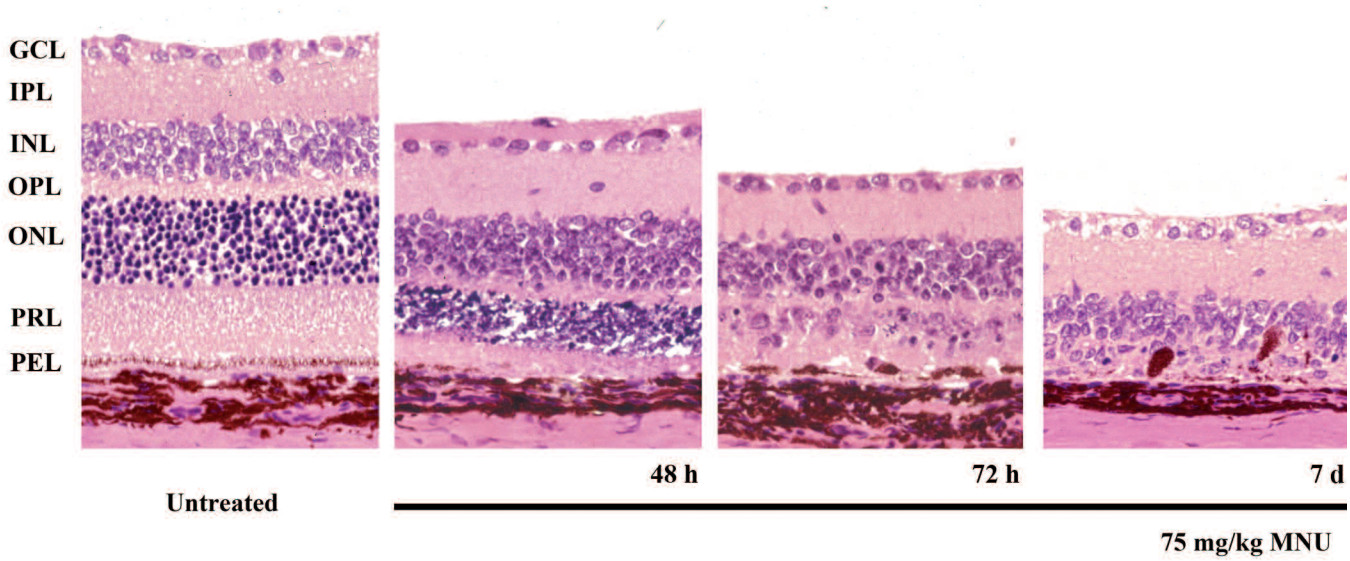

Fig. 1. Time-course progression of retinal change after a single systemic administration of 75 mg/kg N-methyl-N-nitrosourea (MNU) to colored Brown-Norway rats. At 24 and 48 h after MNU administration, photoreceptor cells show pyknosis and karyorrhexis and the destruction of the inner and outer segments. At 72 h, the destruction of photoreceptor cells and photoreceptor segments has progressed, the migration of the melanin-containing retinal pigment epithelial (RPE) cells has began, and mitotic cells among the photoreceptor cells have appeared. At day 7, active signs of photoreceptor cell destruction are indistinct because almost all of the photoreceptor cells have been lost, and the inner nuclear cells are either in direct contact with choroids or separated by a few layers of cells. GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; PRL: photoreceptor cell layer; and PEL: pigment epithelial cell layer.

degeneration) is seen in the inner and outer retina.

Mechanisms of photoreceptor apoptosis caused by MNU

DNA adduct formation

Cell death is the major hallmark of RP, although the mechanisms leading to photoreceptor apoptosis remain poorly understood. MNU is an alkylating agent that interacts with DNA to yield a variety of reaction products. The predominant adduct (70 to 90%) following the reaction of methylating agents with DNA is at the 7-position of guanine, which yields the 7-methyldeoxyguanosine (7-medGua) adduct (Degan et al., 1988). While 7-medGua does not appear to be directly mutagenic, it is useful for the detection of methylating agent exposure because it is more stable than mutagenic O^6-methyldeoxyguanosine. Immunohistochemically, 7-medGua DNA adducts are selectively detected in photoreceptor cell nuclei 6, 12, and 24 h after MNU exposure (Fig. 4), and they gradually decrease at days 3 and 7 (Yoshizawa et al., 1999; Kiuchi et al., 2002). Accumulated DNA adducts in the photoreceptor cell nuclei might suppress DNA synthesis, causing photoreceptor cell death. After DNA adduct accumulation, photoreceptor cell apoptosis is confirmed by DNA laddering, TUNEL signals, and ultrastructural nuclear images.

PARP activity

Poly (ADP-ribose) polymerase (PARP) is an abundant nuclear enzyme involved in DNA repair. DNA damage induced by alkylating agents activates PARP, which repairs DNA damage by using nicotinamide adenine dinucleotide (NAD^+) as a substrate (Carson et al., 1986). PARP hyperactivation results in the depletion of cellular NAD^+ pools, leading to ATP deficiency, energy loss, and subsequent cell death. After MNU administration, the PARP activity in retinas, as evaluated by poly (ADP-ribose) (PAR) expression (the product of PARP), increases at 12 and 24 h and peaks at day 3 (Fig. 5a). PAR immunoreactivity at day 3 is restricted to degenerative photoreceptor cell nuclei (Fig. 5b) (Uehara et al., 2006). Oxidative stress contributes to the pathogenesis of neurodegenerative disorders, including retinal changes in *rd* mice; oxidative stress in these mice is involved in DNA damage (Sanz et al., 2007), which elevates PARP activity and triggers apoptosis-inducing factor (AIF)-mediated photoreceptor cell apoptosis (Paquet-Durand et al., 2007). The activation of PARP plays a pivotal role in mediating photoreceptor cell apoptosis, not only in the MNU model, but also in an inherited mouse model of retinal degeneration. PARP regulates transcription through its interaction with transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) (Chiarugi, 2002; Andreone et al., 2003, Skaper, 2003; Aguilar-Quesada et al., 2007).

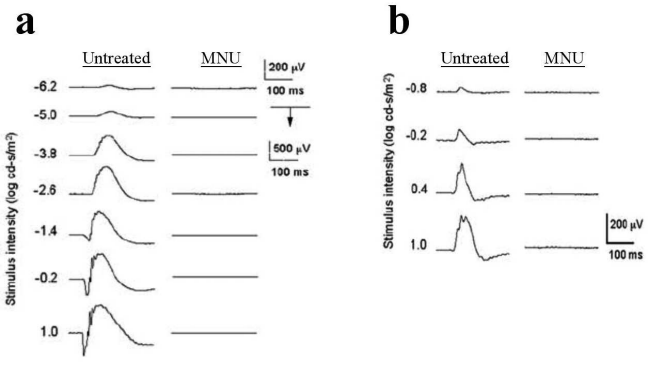

Fig. 2. Electroretinogram (ERG) recorded 7 days after a single systemic administration of 60 mg/kg of *N*-methyl-*N*-nitrosourea (MNU) to albino Sprague-Dawley rats. **a.** Scotopic waveforms. **b.** Photopic waveforms. Both the scotopic and photopic ERG responses were undetectable in the MNU-treated rats.

Transcription factors

NF-κB is a ubiquitous transcription factor that acts as a master regulator of the stress response in various conditions and plays an essential role in cell injury. However, NF-κB activation can induce both anti- and pro-apoptotic effects, depending on the type of cell and the pathological stimulus. NF-κB activity is regulated at multiple levels (Vermeulen et al., 2002). In its latent form in the cytoplasm, NF-κB is complexed with IκB, which prevents its translocation to the nucleus. Phosphorylation of IκB at serine 32 is necessary for the release of active NF-κB. Phosphorylation of the NF-κB p65 subunit at serine 276 is necessary for NF-κB to be constitutively active in the nucleus and exert efficient transcriptional activity. MNU administration does not increase phosphorylated-IκB (serine 32) protein levels. However, phosphorylated-NF-κB (serine 276) protein levels are significantly decreased 12 h after MNU administration (Miki et al., 2007). NF-κB is constitutively active in photoreceptor cells (Krishnamoorthy et al., 1999), and decreased NF-κB activity leads to photoreceptor cell death.

AP-1 is a dimeric complex mainly composed of c-Jun and c-Fos, and it is closely associated with apoptosis. Increased AP-1 activity in the retinas of light-exposed c-$fos^{+/+}$ mice indicates the contribution of AP-1 to apoptosis induction, and c-$fos^{-/-}$ mice retinas resistant to apoptosis show that c-Fos is essential for the light-induced apoptotic pathway (Wenzel et al., 2000). c-Jun and the phosphorylation of its N-terminus at serine 63 and serine 73 by Jun N-terminal kinase (JNK) have been associated with neural apoptosis (Estus et al., 1994; Xia et al., 1995). However, phosphorylated c-Jun is not

required for light-induced photoreceptor apoptosis (Grimm et al., 2001). An analysis of gene expression in the retinas of MNU-treated rats, consisting of microarray analysis and real time RT-PCR, showed up-regulation of c-Fos (Yang et al., 2007). In parallel to apoptosis, the phosphorylation of JNK and c-Jun and the induction of AP-1 (c-Jun and c-Fos) indicate the importance of JNK/AP-1 in the MNU-induced apoptotic pathway (Uehara et al., 2006).

Apoptosis factors

Bcl-2 family proteins act as important determinants of apoptosis; Bcl-2 prevents apoptosis, whereas Bax induces cell death. In the apoptosis cascade, Bcl-2 is down-regulated 12 h after MNU administration, and Bax is up-regulated at 24 h (Yoshizawa et al., 1999). Caspases (cysteine aspartate-specific proteases) coordinate and execute the apoptotic process. The

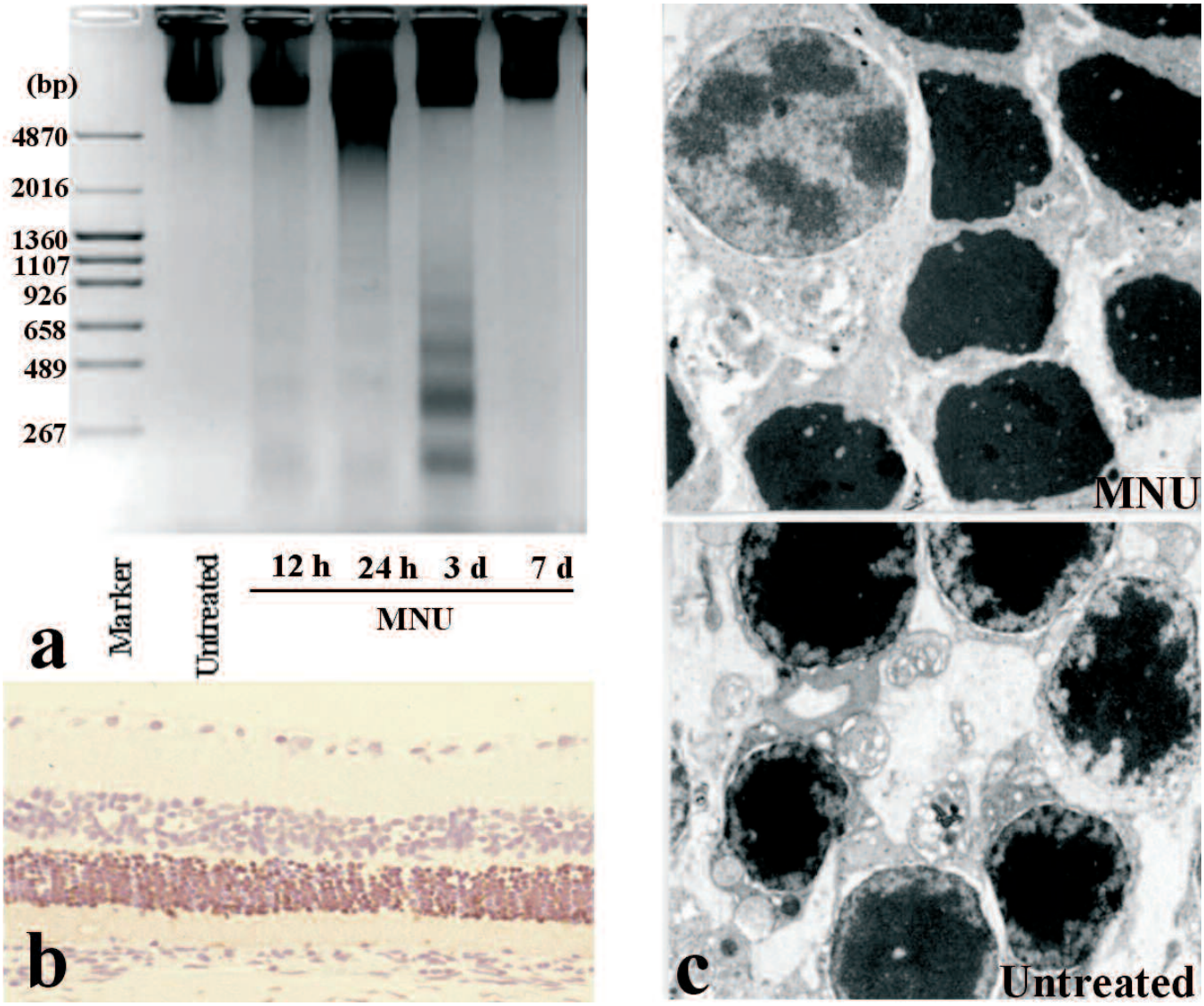

Fig. 3. Apoptotic characteristics of photoreceptor cell death induced by N-methyl-N-nitrosourea (MNU). **a.** DNA fragmentation in the retina of Lewis rats treated with 60 mg/kg MNU and untreated controls. A DNA ladder appeared in the MNU-treated retina after 12 and 24 h and peaked at day 3; the ladder was negligible 7 days after MNU administration. **b.** Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-deoxygenin nick-end labeling (TUNEL). TUNEL-positive signals are restricted to photoreceptor cell nuclei of Sprague-Dawley rats 24 h after administration of 75 mg/kg MNU. **c.** Electron micrograph. MNU-treated rat retina showed hyperchromatic photoreceptor cell nuclei, leaving Müller cells unaffected (see cell at upper left corner). Retinal tissue was collected from Sprague-Dawley rats 24 h after administration of 75 mg/kg MNU. Untreated control photoreceptor cell nuclei are shown in the lower picture.

caspase family plays a decisive role in the execution of retinal apoptosis in that the caspase-3/CPP32, caspase-6/Mch2, and caspase-8/FLICE protease activities peak 72 h after MNU administration (Yoshizawa et al., 1999). Calpains (calcium-dependent cysteine proteases) are activated by increased cellular Ca^{2+} concentrations (Goll et al., 2003). Cytotoxic stimuli results in massive Ca^{2+} influx into the target cells, and PARP activation caused by DNA damage further dysregulates Ca^{2+}, resulting in calpain activation and apoptosis-inducing factor (AIF) induction, which are followed by cell death (Vosler et al., 2009). Total Ca^{2+} in the retinas of MNU-treated rats is significantly increased, and calpain activity is dramatically increased 1 and 3 days after MNU administration and decreased at day 7 (Oka et al., 2007). Calcium overload and activation of calpain activity also occurs in the retinas of *rd* mice (Doonan et al., 2005).

Possible death signals caused by MNU are summarized in Fig. 6. MNU causes DNA adduct formation restricted to photoreceptor cell nuclei, followed by increased PARP activity that leads to the inactivation of NF-κB and activation of JNK/AP-1, causing down-regulation of Bcl-2 and up-regulation of Bax, and the activation of caspase-3, -6, and -8; also, calpain activity is increased by the Ca^{2+} dysregulation. These molecular events may partially explain MNU-induced photoreceptor cell apoptosis. Figure 6 also shows possible strategies for photoreceptor rescue with therapeutic agents.

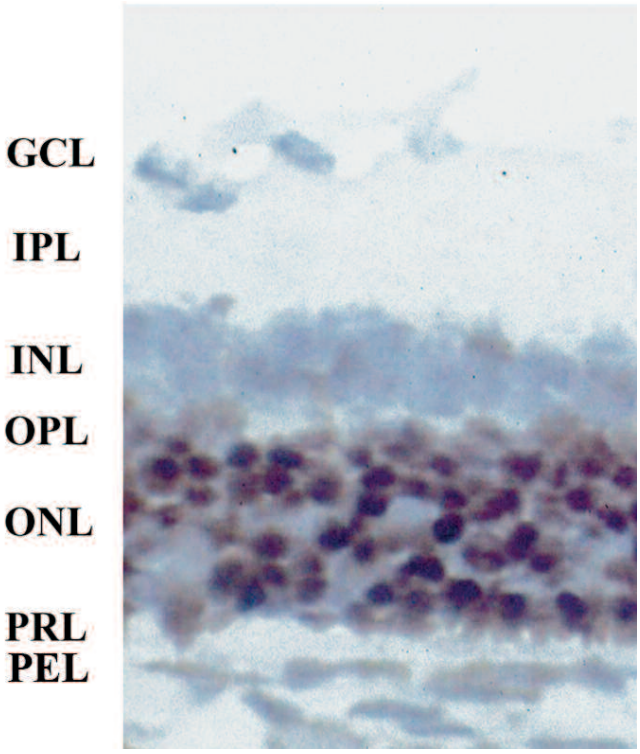

Fig. 4. 7-Methyldeoxyguanosine (7-medGua) DNA adduct formation in the retina of 90-day-old Sprague-Dawley rats 6 h after MNU administration. Positive signals are restricted to photoreceptor cell nuclei. Abbreviations are expanded in the legend for Figure 1.

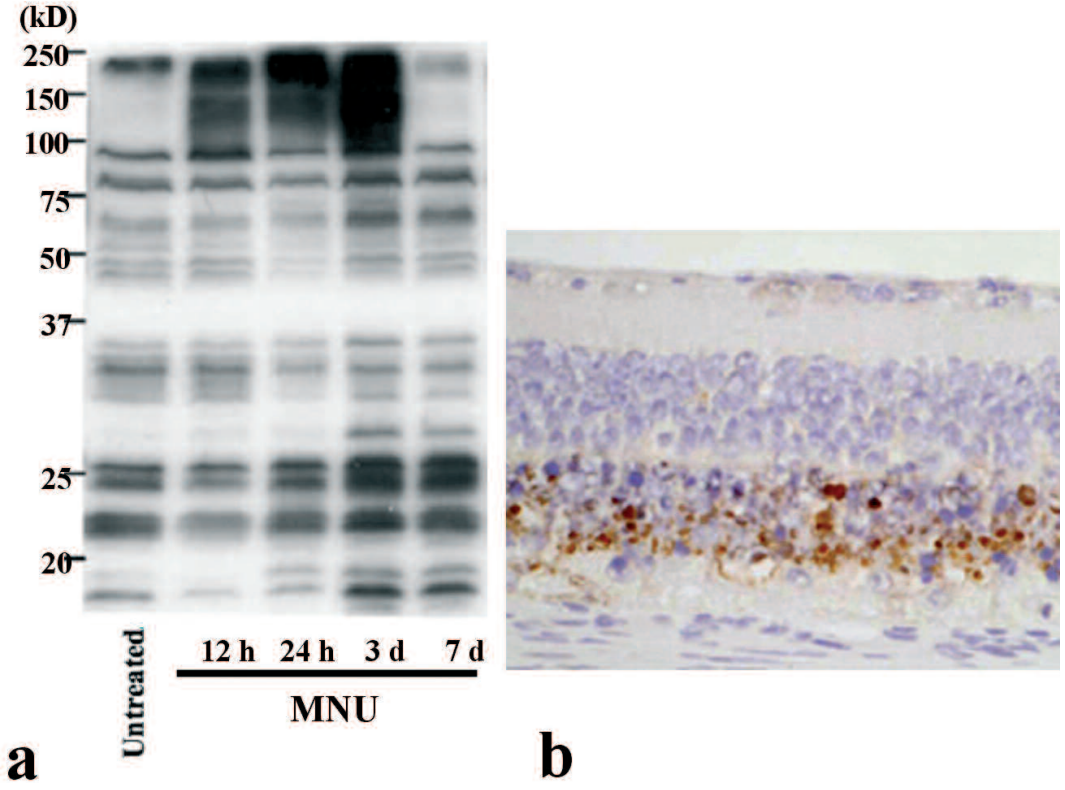

Fig. 5. Poly (ADP-ribose) polymerase activity. **a.** Increased poly (ADP-ribose) (PAR) expression indicates high poly (ADP-ribosylation). Retina samples are from Lewis rats treated with 60 mg/kg MNU and untreated controls. **b.** Immunoreactivity of PAR is selectively seen in degenerative photoreceptor nuclei from the retina of a Lewis rat 3 days after 60 mg/kg MNU administration.

Therapeutic trials against MNU-induced photoreceptor apoptosis

PARP inhibitors

Unless otherwise stated, rats received 60 mg/kg of MNU and were examined 7 days later in the therapeutic trials described below. Nicotinamide (NAM), a water-soluble B group vitamin (vitamin B_3), at a dose of 1000, 250, 50, 25, 10, or 0 mg/kg was subcutaneously injected immediately after MNU administration. NAM suppressed MNU-induced photoreceptor cell loss in a dose-dependent manner (Kiuchi et al., 2002). When NAM was administered at the same time as MNU, photoreceptor cell loss was completely suppressed by ≥25 mg/kg NAM and partially suppressed by 10 mg/kg NAM, as indicated by the photoreceptor cell ratio [(photoreceptor cell thickness / total retinal thickness) x100] in HE-stained sections of the central part of the retina and parallel to the optic axis and nerve, including the ora serrata and optic nerve (Yoshizawa, et al., 2000). The photoreceptor cell ratio in ≥25 mg/kg NAM-treated groups was similar to that of the MNU-untreated control retina, and 1000 mg/kg NAM yielded no side effects. Functionally, scotopic and photopic ERGs showed that 1000 mg/kg NAM protected both rods and cones from MNU damage (Kiuchi et al., 2003). The suppression of photoreceptor cell loss by 1000 mg/kg NAM was confirmed structurally and functionally. Moreover, NAM at a dose of 1000 mg/kg completely suppressed photoreceptor cell loss when administered up to 4 h after MNU and partially suppressed photoreceptor cell loss when administered 6 h after MNU; however, NAM administered 12 h after MNU was ineffective (Kiuchi et al., 2002). NAM did not reduce the levels of 7-medGua DNA adducts. Therefore, NAM did not prevent DNA damage; rather, the DNA damage seemed to be repaired. NAM, a precursor of NAD^+, may block the depletion of NAD^+ or inhibit PARP activation (Purnell and Whish, 1980). When 1000 mg/kg NAM was administered at the same time as MNU, PARP activation was diminished, and the level of down-stream JKN/AP-1 expression was compatible to MNU-untreated animals (Uehara et al., 2006). Therefore, the mechanism by which NAM suppresses MNU-induced retinal damage is PARP inhibition through the JNK/AP-1 signaling pathway. These results indicate that PARP inhibition can block MNU-induced photoreceptor apoptosis. The beneficial effect of PARP inhibition against MNU injury was confirmed by using 3-aminobenzamide (3-AB), which is a PARP inhibitor but not a NAD^+ precursor.

3-AB suppressed MNU-induced photoreceptor apoptosis (Miki et al., 2007). 3-AB (50 mg/kg) subcutaneously injected concurrently with MNU completely suppressed photoreceptor loss, while 30 mg/kg partially suppressed the MNU-induced injury, as determined by the photoreceptor cell ratio. Retinal damage can also be expressed with the retinal damage ratio [(length of damaged retina/whole retinal length) x100]. Damage to the retina was defined as the presence

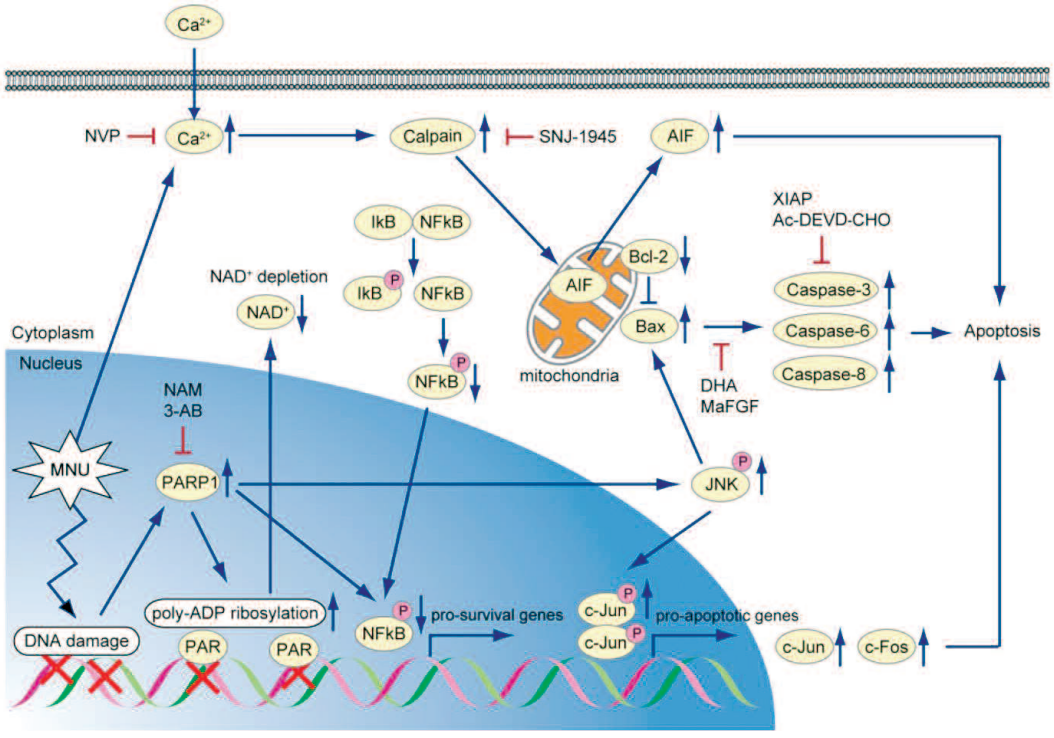

Fig. 6. Possible signaling of MNU-induced photoreceptor apoptosis and therapeutic intervention. NVP: nilvadipine; NAM, nicotinamide; 3-AB: 3-aminobenzamide; DHA: docosahexaenoic acid; MaFGF: mutant of acidic fibroblast growth factor; XIAP: X-linked inhibitor of apoptosis.

of less than four photoreceptor cells in the outer nuclear layer (Yoshizawa, et al., 2000). The retinal damage ratio was 70, 11, and 0% in animals that received 0, 30, and 50 mg/kg 3-AB, respectively. MNU-induced retinal damage proceeds from the central to peripheral retina, indicating that 3-AB suppresses and/or delays the progression of photoreceptor cell damage. In contrast to NAM (Kiuchi et al., 2002), the administration of 3-AB 4 or 6 h after MNU did not rescue photoreceptor damage (Miki et al., 2007). NAM is a PARP inhibitor, a NAD^+ precursor, and a vasodilator (Huang and Chao, 1960). Thus, the two other mechanisms, in addition to PARP inhibition, may also contribute to the retinoprotection provided by NAM. PARP is activated in *rd* retinas, and PARP inhibitors 3,4-dihydro-5-[4-(1-piperidinyl) butoxy]-1"H)-isoquinolinone (DPQ) or 3-AB caused a therapeutic effect in an *ex vivo* model that used *rd* retinal explants (Paquet-Durand et al., 2007). 3-AB treatment preserved NF-κB activity and resulted in photoreceptor cell survival (Miki et al., 2007). Thus, PARP inhibition should be included in RP therapy.

Caspase inhibitors

It is theoretically possible to suppress photoreceptor cell apoptosis by inhibiting the apoptosis cascade. The apoptosis cascade converges on a family of cysteine proteinases known as caspases. X-linked inhibitor of apoptosis (XIAP) inhibits caspases to confer resistance to apoptosis. Subretinal injection of recombinant adeno-associated virus encoding XIAP 6 weeks prior to MNU administration suppressed the MNU-induced photoreceptor apoptosis structurally and functionally throughout the 1-week sampling periods (Petrin et al., 2003). Overall, XIAP-treated eyes showed histologic protection of the outer nuclear layer in 66% (4/6) of the rats at each time point, and XIAP-treated eyes showed a diminished but recordable ERG response in 50% (2/4) of the animals that showed morphologic protection.

Intravitreal administration of Z-YVAD-FMK and Z-DEVD-FMK, caspase-1/4 and 3/7 inhibitors, respectively, along with Z-VAD-FMK, a broad spectrum caspase inhibitor, transiently suppressed photoreceptor apoptosis and preserved photoreceptor function in *RCS* rats (Perche et al., 2008). Z-DEVD-FMK which protects Z-YVAD-FMK showed no protective effect against light-induced retinal degeneration in rats (Perche et al., 2007). Ac-DEVD-CHO, a caspase-3 inhibitor, was intraperitoneally administered to *rd* mice every other day from 8 days of age (Yoshizawa et al., 2002). Photoreceptor cells were significantly preserved in the Ac-DEVD-CHO-treated mice at 13 days of age, but the effect was lost at 17 days of age. Thus, the caspase inhibitor transiently delayed the progression of retinal degeneration in *rd* mice. In rats, two intravitreal administrations of Ac-DEVD-CHO (4000 ng at 0 and 10 h after MNU) suppressed MNU-induced photoreceptor apoptosis 7 days after MNU administration (Yoshizawa et al., 2000). The retinal damage ratio significantly decreased from 99 to 54% by Ac-DEVD-CHO treatment. Therefore, caspase inhibitors are likely to be useful for the treatment of human RP.

Calcium channel blockers and calpain inhibitors

Apoptotic photoreceptors of *rd* mice have increased levels of intracellular calcium (Doonan et al., 2005). In *rd* mice, the calcium channel blockers nilvadipine (NVP) and *cis*- diltiazem (Takano et al., 2004) and the calpain inhibitors N-acetyl-leu-leu-norleucinal (ALLN) and N-acetyl-leu-leu-methioninal (ALLM) reduce photoreceptor apoptosis (Sanges et al., 2006). NVP delays disease progression in *rds* mice (Takeuchi et al., 2008). However, in RCS rats, the calpain inhibitor Mu-Phe-hPhe-FMK (MuhPhe) does not reduce photoreceptor apoptosis (Perche et al., 2008). In MNU-treated rats, 200 mg/kg of calpain inhibitor {(1S)-1-[([(1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl]amino)carbonyl]-3-methylbuyl} carbamic acid 5-methoxy-3-oxapentyl ester (SNJ-1945) orally administered within 30 min after MNU and once daily for 7 days showed a retinoprotective effect (Oka et al., 2007), but NVP was ineffective (Maruyama et al., 2001). The ineffectiveness of NVP in MNU model may be explained by the use of a higher dose of MNU (75 mg/kg, not the typical 60 mg/kg) that might have caused excessive DNA damage in photoreceptor cells that were beyond rescue capacity.

Nutritional factors

Although there are no established standard treatment modalities for RP, vitamin A and docosahexaenoic acid (DHA) supplementation slows the progression of retinal degeneration in RP patients (Shintani et al., 2009). MNU was administered to rats fed the AIN-76A (basal) diet or a DHA diet (5% corn oil and 5% corn starch from the AIN-76A diet is replaced by 9.5% DHA and 0.5% linoleic acid (LA) in weight percentages) for 2 weeks; then, the rats continued to receive the same diet or were switched to the opposite diet until the termination; rats were sacrificed 3 or 7 days after MNU administration (Moriguchi et al., 2004). Three days after MNU administration, the progression of photoreceptor damage was delayed in parallel with serum DHA levels; seven days after MNU administration, although the damage was not delayed in parallel with serum DHA levels, the retinal damage ratio was significantly lower in rats fed DHA at some point than in rats fed the basal diet throughout the experimental period. The retinal damage ratio was 38% in rats which received the DHA diet for 14 days before MNU administration, 46% in rats which received the DHA diet 7 days after MNU administration, and 47% in rats which received the DHA diet 14 days before and 7 days after MNU administration; the retinal damage ratio was 88% in rats which received the basal AIN-76A diet (without DHA) throughout the experimental period. Thus, although the DHA diet did not completely counteract the MNU stimulus, it likely

delayed the onset and suppressed the progression of photoreceptor cell apoptosis. DHA interferes with pro- and anti-apoptotic proteins of the Bcl-2 family and protects photoreceptor cells from apoptosis (Rotstein et al., 2003). Rats were given MNU at a dose of 50 mg/kg, which is lower than the dose needed to cause photoreceptor cell loss within 7 days in all treated animals, and then one of five diets containing the following fatty acids: 10% LA (LA diet); 9.5% palmitic acid (PA) and 0.5% LA (PA diet); 9.5% eicosapentaenoic acid (EPA) and 0.5% LA (EPA diet); 4.75% EPA, 4.75% DHA and 0.5% LA (DHA plus EPA diet); or 9.5% DHA and 0.5% LA (DHA diet). The rats were observed for 20 weeks after MNU administration (Moriguchi et al., 2003). The degree of retinal lesions varied significantly among the 5 diet groups. The incidence [(number of rats with retinal degeneration / total number of rats) x 100] and retinal damage ratio for each diet was: LA diet, 88 and 61%; PA diet, 41 and 18%; EPA diet, 73 and 40%; EPA plus DHA diet, 53 and 24%; and DHA diet, 0 and 0%, respectively. The DHA diet completely suppressed retinal damage, but the EPA (which is another n-3 polyunsaturated fatty acid (PUFA) like DHA) diet did not suppress retinal damage. The DHA plus EPA diet had a weaker retinoprotective effect, whereas the lesions were accelerated in rats fed the LA (a member of n-6 PUFA) diet.

Neurotrophic factors

Neurotrophic factors protect neurons and initiate their growth. These factors are neuroprotective in a range of diseases including retinal disease (Thanos and Emerich, 2005). Acidic fibroblast growth factor (aFGF) ameliorates ischemic injury of the rat retina (Cuevas et al., 1998), but the use of aFGF requires caution because of its mitogenic activity (Galzie et al., 1997). In this context, mutant aFGF (MaFGF), which is a non-mitogenic form of human aFGF, has been developed and applied in the MNU system (Wu et al., 2005) by intravitreal injections at 0 and 12 h after MNU administration (Xu et al., 2008). Intravitreal administration of MaFGF at a dose of 1.25 and 2.5 μm partially protected MNU-induced retinal damage in rats by up-regulating Bcl-2 and down-regulating Bax.

Cell transplantation

In addition to the pharmacological approaches described above, degenerative photoreceptors can be repaired and retinal function rescued if the damaged photoreceptors can be replaced with healthy cells that make appropriate synaptic connections with the remaining functional circuitry within the retina. Photoreceptor cells and/or stem/progenitor cells can be used for cell transplantation. Photoreceptor cell loss caused by MNU can be repaired by cell transplantation. Synapses between transplanted neurons and host retina were built, and partial functional recovery was achieved when dissociated photoreceptors were transplanted concomitantly with chondroitinase ABC (ChABC), a bacterial enzyme that degrades the chondroitin sulfate proteoglycan (CSPG) side chain, one day after MNU treatment in mice (Suzuki et al., 2007). ChABC, which degrades CSPGs in the extracellular matrix, may help to eliminate the barrier between photoreceptor transplants and host retina and may increase the neurite-extending activity. Some of the progeny of the Müller glia in adult rat retina undergo gliosis and proliferate in response to photoreceptor cell loss caused by MNU, and they express a rod-specific marker and synaptic protein (Wan et al., 2008). Cultured Müller cells that are transplanted show a regenerative capacity by migrating into the MNU-induced damaged retina and expressing a rod-

Table 2. Percent inhibition of retinal damage after therapeutic trials of different agents against MNU injury in rats.

Reagent	Dose	Frequency or Duration	Route	Retinal damage ratio (%)		Photoreceptor cell ratio (%)		Reference
				Treated/Untreated	Inhibition (%)	Treated/Untreated	Inhibition (%)	
NAM	1000 mg/kg·BW	x1	sc			33/6	82	Kiuchi et al., 2002
	25 mg/kg·BW	x1	sc			34/6	82	
	10 mg/kg·BW	x1	sc			22/6	73	
3-AB	50 mg/kg·BW	x1	sc	0/70	100	48/18	63	Miki et al., 2007
	30 mg/kg·BW	x1	sc	11/70	84	38/18	53	
AC-DEVD-CHO	4000 ng	x2	iv	54/99	45	12/3	75	Yoshizawa et al., 2000
DHA	9.5g/100g·diet	14 days[1)]	po	38/88	57			Moriguchi et al., 2004
	9.5g/100g·diet	7 days[2)]	po	46/88	48			
	9.5g/100g·diet	21 days[3)]	po	47/88	47			

Retinal damage ratio is (retinal length composed of less than four rows of photoreceptor cells / total retinal length) x 100. Photoreceptor cell ratio at the posterior pole is (photoreceptor cell thickness / total retinal thickness) x 100. Percent inhibition of retinal damage is calculated as the retinal damage ratio in [(Untreated – Reagent-treated animals) / Untreated animals] x 100 or the photoreceptor cell ratio in [(Reagent-treated – Untreated animals) / Reagent-treated animals] x 100. Seven-week-old female Sprague-Dawley rats were injected intraperitoneally with 60 mg/kg MNU and sacrificed 7 days later. 1) before MNU; 2) after MNU; 3) 14 days before and 7 days after MNU. NAM: nicotinamide; 3-AB: 3-aminobenzamide; Ac-DEVD-CHO: caspase-3 inhibitor; DHA: docosahexaenoic acid; BW: body weight; sc: subcutaneous; iv: intravitreal; po: peroral.

specific marker. Müller cells have progenitor/stem cell properties and can be used for cell replacement therapy.

Concluding remarks

A single administration of MNU induces photoreceptor cell loss in various animal species. Disease progression is rapid in that the active signs of photoreceptor cell apoptosis have ended by day 7. MNU causes DNA adduct formation in photoreceptor nuclei, followed by increased PARP activity that leads to the activation of JNK/AP-1 and inactivation of NF-κB, causing the down-regulation of Bcl-2, up-regulation of Bax, and the activation of caspase-3, -6, and -8. In addition, increased intracellular calcium levels activate calpain. Collectively, these molecular events appear to cause photoreceptor cell apoptosis. Many novel approaches have been applied to control MNU-induced photoreceptor cell death (see Fig. 6). MNU-induced photoreceptor apoptosis can be suppressed by the following agents: PARP inhibitors, such as NAM and 3-AB; DHA and MaFGF, which may interfere with pro- and anti-apoptotic molecules; caspase inhibitors XIAP and Ac-DEVD-CHO; and calpain inhibitor SNJ-1945. In addition to pharmacological manipulation, cell transplantation may possibly rescue MNU-induced retinal damage. The potency of agents that suppress photoreceptor cell loss is difficult to compare. Comparisons made with the same model system and evaluation method are listed in Table 2. In MNU-exposed rats, the percent inhibition of retinal damage is calculated by the retinal damage ratio and/or the photoreceptor cell ratio. Although different evaluations with different model systems may yield different results, these trials may be suitable for human studies.

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