Abstract. Aim: Retinitis pigmentosa is a group of inherited neurodegenerative human diseases characterized by the loss of photoreceptor cells by apoptosis and lead to eventual blindness. A single intraperitoneal (i.p.) injection of N-methyl-N-nitrosourea (MNU), an alkylating agent, causes photoreceptor cell apoptosis within seven days in rats. Curcumin is a polyphenolic natural product with pluripotent properties including antioxidant activity. The purpose of the present study was to evaluate the efficacy of curcumin against photoreceptor apoptosis in a MNU-induced retinal degeneration rat model. Materials and Methods: Seven-week-old female Sprague-Dawley rats received a single i.p. injection of 40 mg/kg MNU. Three days prior to MNU injection, daily i.p. injections of 100 or 200 mg/kg curcumin were started, and the injections were continued once daily until sacrifice. Rats were sacrificed at 6, 12, 24 and 72 h, and 7 days after MNU, and their eyes were examined morphologically and morphometrically to evaluate the photoreceptor cell ratio and retinal damage ratio in hematoxylin and eosin-stained sections. Retinal 8-hydroxy-2-deoxyguanosine (8-OHdG) levels were quantified by enzyme-linked immunosorbent assay (ELISA), and the apoptotic cell ratio in photoreceptor cells was determined in situ by TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL). Results: Curcumin (200 mg/kg) significantly \((p<0.01)\) suppressed the loss of photoreceptor cells, as determined by the photoreceptor cell ratio at the central retina seven days after MNU, and this effect was dose-dependent. At 12 h after MNU injection, when the oxidative DNA damage caused by MNU peaked, curcumin significantly reduced the level of 8-OHdG (0.78 vs. 0.50 ng/ml) \((p<0.05)\) and the percentage of TUNEL-positive photoreceptor cells (17.5% vs. 10.8%) \((p<0.05)\) as compared with MNU-exposed, curcumin-untreated retina, respectively. Conclusion: Curcumin inhibited MNU-induced photoreceptor cell apoptosis by suppressing DNA oxidative stress. These findings indicate that curcumin may help to suppress the onset and progression of human retinitis pigmentosa.

Retinitis pigmentosa (RP) is characterized by early night blindness followed by peripheral visual field alterations (known as tunnel vision) and eventual blindness. Clinical symptoms typically start in the early teenage years, and severe visual impairment occurs by 40-50 years of age. The estimated incidence of RP is one in 4,000, which makes it one of the most common causes of severe visual impairment in humans (1). Rods in the retina, which provide black-and-white vision and function in dark or dim light, are the first photoreceptors to be lost. The loss of rods initially occurs in the equatorial zone and then extends peripherally and centrally. Cones, which are responsible for color vision and function in bright light, are lost after the rods. RP is a heterogeneous group of retinal disorders with autosomal dominant inheritance, autosomal recessive inheritance, or X-linked inheritance. RP is a non-inflammatory, bilateral, progressive, degenerative retinopathy of genetic origin, and more than 160 different mutations in genes that encode proteins with remarkably diverse functions result in rod photoreceptor degeneration (www.sph.uth.tmc.edu/retnet). Although various genes are involved in RP, the final common pathway is apoptotic cell death of the rod photoreceptors (2). Vitamin A (3), vitamin A
in combination with docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid (PUFA) found in fish (4), and lutein found in green leafy vegetables (5) can slow the progression of human RP. However, there are currently no effective drugs for RP; thus, the identification of chemicals that are effective against RP is needed.

Spontaneous and transgenic animal models for RP are important to gain a better understanding of human RP and to establish possible therapies for it (6, 7). N-Methyl-N-nitrosourea (MNU), an alkylating agent, exhibits cytotoxicity by transferring its methyl group to nucleobases in nucleic acids. MNU selectively damages photoreceptor cells and is a good candidate for the induction of photoreceptor degeneration. MNU-induced photoreceptor degeneration is due to selective 7-methyldeoxyguanosine DNA adduct formation in photoreceptor nuclei, followed by photoreceptor cell death via an apoptotic mechanism, similar to the mechanism of human RP (8).

Oxidative stress contributes to the pathogenesis of neurodegenerative disorders, and the production of reactive oxygen species (ROS) in response to oxidative stress is implicated in photoreceptor cell death, including retinal DNA damage in retinal degeneration animal models, such as rd mice (9). When 100 μg/ml of MNU are added to murine 661W photoreceptor-derived cells or mouse RGC-5 retinal ganglion cell lines in culture, MNU induces radical generation (as measured by the intracellular ROS levels) in the 661W photoreceptor cells but not in the intracellular ROS levels in the 661W photoreceptors and in the RGC-5 retinal ganglion cells (10). Thus, MNU selectively elevates ROS in photoreceptor cells and induces photoreceptor cell death in vitro.

Traditional medicines provide pharmacotherapy for people worldwide. Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a naturally-occurring yellow pigment of turmeric and constituent of curry powder that is isolated from the rhizomes of Curcuma longa (Linn) and is commonly used in Asian cooking as a coloring and flavoring agent. Curcumin has also been used in both Oriental and Ayurvedic medicine since ancient times (11). Curcumin has beneficial properties that include antioxidant activity (12-14). Furthermore, curcumin crosses the blood brain and blood retinal barriers (15, 16). Thus, theoretically, curcumin may ameliorate photoreceptor cell damage caused by MNU. The present study was designed to explore the efficacy of curcumin against photoreceptor cell damage caused by MNU in Sprague-Dawley rats.

Materials and Methods

Animals. Six-week-old female Sprague-Dawley rats [Crl:CD (SD)] were purchased from Charles River Japan (Osaka, Japan). Animals were housed in groups of four or five in plastic cages with paper bedding (Paper Clean, SLC, Hamamatsu, Japan) in a specific pathogen-free conditioned room maintained at 22±2°C and 60±10% relative humidity with a 12-h light/dark cycle (lights on from 8:00 am to 8:00 pm; illumination intensity less than 60 lux at the cage level). Animals were maintained on a commercial pellet diet (CMF 30 kGy; Oriental Yeast, Chiba, Japan) and had free access to water. After a one-week acclimatization period, experiments were begun when rats were seven weeks of age. The study protocol and animal procedures were approved by the animal care and use committee of Kansai Medical University (permit number: 12-078). Throughout the experiments, animals were cared for in accordance with the Guidelines for Animal Experimentation of Kansai Medical University.

Chemicals. MNU in a powder form was obtained from Sigma-Aldrich (St. Louis, MO, USA) and stored at 4°C in the dark. A 5 mg/ml solution was prepared by dissolving MNU in physiological saline containing 0.05% acetic acid immediately before use. A powdered form of curcumin was purchased from Sigma-Aldrich and suspended at 20 mg/ml in physiological saline.

Experimental procedures. Female rats received a single intraperitoneal (i.p.) injection of 40 mg/kg MNU at seven weeks of age. A daily dose of 100 or 200 mg/kg curcumin was administered i.p. starting three days prior to MNU administration, and the injections were continued once daily until sacrifice (Figure 1). Doses of 100 and 200 mg/kg curcumin were selected based on a previous report (17). Control rats received an equivalent volume of saline instead of curcumin at the same time points. Rats were divided into the following groups: MNU-unexposed and curcumin-treated (MNU+/CUR+); MNU-unexposed and 200 mg/kg curcumin-treated (MNU+/200 CUR+); MNU-exposed and curcumin-treated (MNU+/CUR+); and MNU-exposed and 100 or 200 mg/kg curcumin-treated (MNU+100 or 200 CUR+). When only a dose of 200 mg/kg curcumin (but not 100 mg/kg curcumin) was used with MNU-exposed rats, the group was designated as MNU+/CUR+.

On the day of MNU exposure, curcumin was administered 2 h prior to MNU injection. All rats were observed daily for clinical signs of toxicity and were weighed on the day of MNU administration and on the day of sacrifice. At the time of sacrifice, both eyes were quickly removed from each animal for examination.

Tissue fixation and processing. One eye was fixed overnight in 10% neutral buffered formalin or methacarn (60% methanol, 30% chloroform and 10% acetic acid), and the other eye was used for DNA purification. Formalin- or methacarn-fixed samples were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE). Sections including the ora serrate and optic nerve were used for the evaluation. Formalin-fixed tissues were used to detect cell death by TdT-mediated dUTP-digoxigenin nick-end labeling (TUNEL) staining, and methacarn-fixed tissues were used for morphologic and morphometric analyses.

Morphometric analysis of photoreceptor cell ratio and retinal damage ratio. Methacarn-fixed and HE-stained ocular sections obtained 6, 12, 24 and 72 h and seven days after MNU or without MNU were used. Sections were obtained from the MNU+/CUR-, MNU-/200 CUR+, MNU+/CUR-, MNU+/100 CUR+, and MNU+/200 CUR+ groups. As described previously (18), NDPview was used to measure the total retinal thickness (from the internal limiting membrane to the retinal pigment epithelial cells) and photoreceptor thickness (the outer nuclear layer and the inner and outer segments). The measurements were taken from the peripheral retina (approximately 400 μm from both sides of the ciliary body).
In situ detection of apoptosis.

Formalin-fixed ocular sections (LOQ) for 8-OHdG in ELISA was 0.125 and 10 ng/ml, respectively. Concentration was calculated from a standard curve and corrected for absorbance was then measured at 450 nm. Tissue sample developed with the addition of 3,3',5,5'-tetramethylbenzidine, microtiter plate precoated with 8-OHdG. After the final color was overnight at 4˚C with a monoclonal antibody against 8-OHdG in a 8-OHdG standard or DNA purified from the retina was incubated a Aging, Fukuroi, Japan) according to the manufacturer's protocol. The Sensitive 8-OHdG Check ELISA; Japan Institute for the Control of immnosorbent assay (ELISA) with a DNA oxidation kit (Highly Osaka, Japan) according to the manufacturer's instructions. Each group consisted of 4-7 retinas, and one retina per rat was collected. The retinal 8-OHdG levels were measured by enzyme-linked immnosorbent assay (ELISA) with a DNA oxidation kit (Highly Sensitive 8-OHdG Check ELISA; Japan Institute for the Control of Aging, Fukuroi, Japan) according to the manufacturer's protocol. The 8-OHdG standard or DNA purified from the retina was incubated overnight at 4˚C with a monoclonal antibody against 8-OHdG in a microtitr plate precoated with 8-OHdG. After the final color was developed with the addition of 3,3',5,5'-tetramethylbenzidine, absorbance was then measured at 450 nm. Tissue sample concentration was calculated from a standard curve and corrected for DNA concentration. The limits of detection (LOD) and quantification (LOQ) for 8-OHdG in ELISA was 0.125 and 10 ng/ml, respectively.

In situ detection of apoptosis, Formalin-fixed ocular sections obtained 6, 12, and 24 h after MNU injection were used. Sections were obtained from the MNU+/CUR−, MNU−/CUR+, MNU+/*CUR−, and MNU+/CUR+ groups (200 mg/kg CUR+ group). Apoptosis was observed by TUNEL staining with an in situ apoptosis detection kit (TACS2 TdT; Trevigen, Gaithersburg, MD, USA). The reaction products were visualized with 3,3'-diaminobenzidine as chromogen. Measurements were made from the central retina (approximately 400 μm from the optic nerve), and the TUNEL-positive cell ratio was calculated as [(number of TUNEL-positive photoreceptor cells/500 photoreceptor cells) ×100].

Statistics. All discrete values were expressed as the mean±S.E. and were analyzed by using the one-tailed independent t-test for paired or unpaired samples after assuring the homogeneity of variances using Excel Statistical Program File ystat 2008.xls. p-Values of <0.05 were accepted as being statistically significant.

Results

General remarks. No deaths occurred, and no clinical signs or symptoms were evidenced during the experimental period. However, the growth rate of MNU-exposed rats tended to be lower than that of not exposed to MNU rats (data not shown).

Morphological and morphometric analyses. Retinal histology showed no abnormal changes in the central or peripheral retina in MNU+/CUR− and MNU−/200 CUR+ rats at seven days; thus, the administration of 200 mg/kg curcumin caused no retinal side-effects (Figure 2). In the central retina of MNU+/CUR− rats, the outer nuclear layer and the photoreceptor layer disappeared or were reduced to a few rows of photoreceptor cell nuclei seven days after MNU injection. In contrast, in the rats of the MNU+/100 CUR+ and MNU+/*200 CUR+ groups, curcumin dose-dependently ameliorated the loss of the outer nuclear layer and photoreceptor cell layer in the central retina as compared to MNU+/CUR− rat retina. To further quantify the effect of curcumin on retinal thickness, the photoreceptor cell ratio at the central retina was used for comparison. Although 200 mg/kg curcumin did not completely ameliorate photoreceptor cell loss when compared with MNU+/CUR− rat retina, from rats of the MNU+/200 CUR+ group had a statistically significant amelioration of photoreceptor cell survival as compared to those from MNU+/CUR− and MNU+/100 CUR+ groups (Figure 3a). As MNU-induced retinal degeneration in rats begins in the central retina and progresses to the peripheral retina, the change in peripheral retina in MNU-exposed rats seven days after MNU injection was similar among groups when compared using the photoreceptor cell ratio. When the retinal damage ratio was used for comparison, retinal damage in MNU+/200 CUR+ rats tended to be mitigated as compared to that in MNU+/CUR− and MNU+/100 CUR+ rats, although the value did not reach statistical significance (Figure 3b).

Sequential changes in retinal damage. The central and peripheral retinal portions of MNU+/CUR−, MNU+/CUR+, MNU+/CUR−, and MNU+/CUR+ rats (200 mg/kg curcumin) were compared 72 h and seven days after 40 mg/kg MNU
injection (Figure 4). The central retina contained pyknotic nuclei in the photoreceptor cells 72 h after MNU injection, and apoptotic cell loss was observed in the central retina seven days after MNU injection. The retinal damage extended from the central retina towards the peripheral retina but did not completely reach the peripheral retina seven days after MNU injection. The photoreceptor cell ratios 72 h after MNU injection in MNU−/CUR−, MNU−/CUR+, MNU+/CUR−, and MNU+/CUR+ rats were 47.5%, 44.6%, 27.0%, and 28.9% for the central retina and 52.9%, 55.1%, 48.6%, and 48.3% for the peripheral retina, respectively (Figure 4a). The corresponding ratios seven days after MNU injection were 47.0%, 48.8%, 5.3%, and 23.2% for the central retina and 52.2%, 54.6%, 42.9%, and 42.7% for the peripheral retina, respectively (Figure 4b). MNU-induced retinal damage with or without curcumin administration was similar 72 h after MNU injection. However, when retina from rats of the MNU+/CUR+ group was compared with that of the MNU+/CUR− group seven days after MNU injection, 200 mg/kg curcumin significantly suppressed MNU-induced damage in the central retina, but the values in the peripheral retina were similar.

**Suppression of retinal 8-OHdG levels.** Retinal generation of 8-OHdG, a marker of oxidative DNA damage, was measured by ELISA (Figure 5). Retinal 8-OHdG levels in MNU+/CUR− and MNU+/CUR+ rats at 6, 12, and 24 h after MNU injection were 0.55 and 0.51 ng/ml; 0.78 and 0.50 ng/ml ($p<0.05$); and 0.63 and 0.52 ng/ml; respectively. The retinal 8-OHdG levels in MNU+/CUR− and MNU+/CUR+ rats at 24 h were 0.58 and 0.53 ng/ml, respectively. After MNU injection, retinal 8-OHdG levels increased and peaked at 12 h. Retinal 8-OHdG levels in MNU+/CUR+ rats were significantly reduced compared with MNU+/CUR− rats and were comparable to those of rats not exposed to MNU. Therefore, the oxidative stress was significantly suppressed by 200 mg/kg curcumin.

**Apoptotic cell ratio.** The apoptotic cell ratio was evaluated in the central retina. In retina from rats not exposed to MNU (MNU−/CUR− and MNU−/CUR+), apoptotic cells were not detectable at any examined time point, neither with nor without curcumin treatment; thus, curcumin did not cause apoptotic cell death in photoreceptor cells. The sequential changes in the apoptotic cell ratio in retina from rats of the MNU+/CUR− and MNU+/CUR+ groups at 6, 12, and 24 h after MNU injection were 0% and 0%, 17.5% and 10.8% ($p<0.05$), and 22.1% and 29.5% (not significant), respectively; thus, 200 mg/kg curcumin significantly lowered the TUNEL-positive ratio 12 h after MNU injection when the oxidative stress peaked (Figure 6).

**Discussion**

The MNU-induced animal model for human RP is a valuable model in searching for possible chemicals suitable for photoreceptor rescue. By using this model, inhibitors of poly(ADP-ribose) polymerase, caspase, calpain, and ROS, and natural chemicals, such as docosahexaenoic acid, arachidonic acid, and reishi were found to be effective for photoreceptor rescue (8).

In the present study, *i.p.* administration of 40 mg/kg MNU to Sprague-Dawley rats caused time-dependent retinal damage in which photoreceptor cell loss progressed over a seven-day course. Although retinal damage was similar 72 h
after MNU injection, 200 mg/kg curcumin significantly suppressed photoreceptor cell loss seven days after MNU injection, as evaluated morphologically and morphometrically by using the photoreceptor cell ratio at the central retina but not the peripheral retina. The degeneration caused by MNU originates from the central retina (posterior pole) and extends to the periphery; with the MNU dose regimen used in the present study, the disease progression did not completely reach the peripheral retina seven days after MNU injection. However, 200 mg/kg/day of curcumin effectively (although not completely) suppressed the retinal damage caused by MNU. This report is the first, to our knowledge, to show that the beneficial effects of curcumin in the MNU-induced animal model of human RP are due to a reduction of oxidative stress, which is important in the development of RP (19). These results raise the possibility that curcumin may help inhibit the development of human RP.

 Beneficial therapeutic effects with minimal toxicity have been found for natural phytochemicals. Curcumin is a phytochemical that is a promising therapeutic candidate for retinal diseases (20). Phase I clinical trials indicate that curcumin is well-tolerated when taken at doses as high as 12 g/day (21). The European Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources concluded that the present database supports an acceptable daily intake of 3 mg/kg based on the no observed adverse effect level of

Figure 3. Quantitative analysis of the effects of curcumin (CUR) on N-methyl-N-nitrosourea (MNU)-induced retinal damage. Retinal changes after 40 mg/kg MNU exposure and the effect of 100 or 200 mg/kg CUR in comparison to MNU+/CUR– control retina. a: Quantitative analysis of retinal damage as evaluated by the photoreceptor cell ratio: [(photoreceptor thickness/total retinal thickness) × 100] at the central and peripheral retina seven days after a single i.p. injection of MNU. Each group consisted of five retinas. Note the dose-dependent effect of CUR in suppressing retinal damage at the central retina. b: Quantitative analysis of retinal damage as evaluated by the retinal damage ratio: [(retinal length composed of less than four rows of photoreceptor cells/total retinal length) × 100] seven days after a single i.p. injection of MNU. Each group consisted of five retinas. Note that the retinal damage ratio was reduced by 200 mg/kg CUR, but the reduction did not reach statistical significance. MNU+/CUR– vs. MNU+, and MNU+ between groups, respectively, were statistically compared. Data are the mean±S.E. **p<0.01,*p<0.05.
250-320 mg/kg body weight/day from a repeated-dosing toxicity study in rodents (22). Therefore, in the present study, 200 mg/kg/day of curcumin was selected as a safe and available dose. Dietary supplementation of 200 mg/kg curcumin every day for 20 weeks is well tolerated and does not cause death of Wistar rats (23). In the present study, daily injection of 200 mg/kg curcumin i.p. for 10 times to Sprague-Dawley rats caused no side-effects.

Curcumin protected against N-methyl-D-aspartate (NMDA)-induced toxicity in primary retinal cells in culture (24) and oral supplementation of curcumin provided functional and structural protection of photoreceptor cells against light-induced damage in rats (25) and of photoreceptor degeneration in transgenic rats with the P23H rhodopsin mutation (15). Rhodopsin is a biological pigment in photoreceptor cells, and the P23H missense mutation in rhodopsin accounts for the majority of cases of autosomal-dominant RP (26). In addition to the protection provided to photoreceptor cells, curcumin protects retinal ganglion cells against ischemia and reperfusion injury (20) and staurosporine-mediated injury (27). Curcumin also protects retinal pigment epithelial cells against H2O2 cytotoxicity (16). Thus, curcumin protects against retinal cell damage caused by different agents, including retinal damage to photoreceptor cells.

Figure 4. Sequential and quantitative analysis of the effect of curcumin (CUR) on N-methyl-N-nitrosourea (MNU)-induced retinal damage. Retinal changes after 40 mg/kg MNU exposure and effect of 200 mg/kg CUR in comparison to MNU+/CUR− control retina, and comparison of CUR effect on MNU-exposed rat retina at 72 h and seven days after MNU administration. a: Quantitative analysis of retinal damage at 72 h as evaluated by the photoreceptor cell ratio at the central and peripheral retina. Each group consisted of five retinas. b: Quantitative analysis of retinal damage at seven days after MNU administration as evaluated by the photoreceptor cell ratio at the central and peripheral retina. Each group consisted of five retinas. Note that 200 mg/kg CUR significantly, but not completely, suppressed retinal damage at the central retina. MNU+/CUR− vs. MNU+, and MNU+/CUR− vs. MNU+/CUR+, respectively, were statistically compared. Data are the mean±S.E. **p<0.01.
Oxidative stress is implicated in various eye diseases. 8-OHdG is one of the most abundant oxidative products of DNA damage and represents a sensitive biomarker of oxidative stress. Curcumin is a potent scavenger of ROS, such as superoxide anion, hydroxyl radical, singlet oxygen, nitric oxide, and peroxynitrite, and reduction of ROS can prevent the formation of 8-OHdG (23). In diabetic retinopathy, dietary supplementation with 0.05% curcumin prevents streptozotocin-induced diabetes and decreases diabetes-induced accumulation of oxidative products (reflected by 8-OHdG levels) in the retina (28). MNU induces free-radical production in cultured murine 661W photoreceptor-derived cells, and 1 μM edaravone (EDRV), a potent antioxidant and free-radical scavenger, reduces MNU-induced free-radical production (10). In ddY mice, when 0, 0.3, or 1.0 mg/kg EDRV was injected intravenously immediately and 6 h after 60 mg/kg MNU treatment, EDRV at 1.0 mg/kg (but not 0.3 mg/kg) significantly suppressed the reduction in the outer nuclear thickness as compared with mice not treated with EDRV seven days after MNU. Immunohistochemically, the number of MNU-induced 8-OHdG-positive photoreceptor cell nuclei was significantly reduced by 200 mg/kg curcumin, resulting in a significant decrease in TUNEL-positive cells, which resulted in the significant rescue of photoreceptor cells seven days after MNU injection. Although very few clinical studies of curcumin for eye diseases have been reported, curcumin has been tested in anterior uveitis and central serous chorioretinopathy but not in RP (20). As curcumin is non-toxic, it may be a potent candidate in treating human RP.

Acknowledgements

We are grateful to Ms. T Akamatsu for her technical help and Ms. A Shudo for the manuscript preparation. Authors have no competing financial interests. This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japanese Society for the Promotion of Science (24592661, 25462740).

References


EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS): Scientific Opinion on the reevaluation of curcumin (E 100) as a food additive. EFSA J 8: 1679, 2010.