Age-specific and Dose-dependent Retinal Dysplasia and Degeneration Induced by a Single Intraperitoneal Administration of N-Methyl-N-nitrosourea to Rats

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Abstract: The morphologic response of the rat retina at different ages to the various doses of the alkylating agent N-methyl-N-nitrosourea (MNU) was examined. MNU at the dose of 35, 50, 75, 90, and/or 105 mg/kg was injected intraperitoneally (ip) to male and female Sprague-Dawley and Lewis rats at 0, 21, 50, and 150 days of age, and the retinas were examined 7 or 21 days after the treatment. In both strains of rats, regardless of gender, MNU evoked age-specific and dose-dependent retinal lesions. When ≥ 50 mg/kg MNU were administered at day 0, retinal dysplasia characterized by rosette formation was found in the outer neuroblastic/nuclear layer above the continuous lining single layer of pigment epithelial cells. The severity of the lesion was not dose-related. When >90 mg/kg, >50 mg/kg and ≥ 35 mg/kg MNU were administered at day 21, 50, and 150, respectively, retinal degeneration characterized by selective photoreceptor cell loss due to apoptosis with disorganized pigment epithelial cells was found in all rats. Thus, the sensitivity of photoreceptor cells in the development of retinal degeneration induced by MNU increased in parallel with aging. (*J Toxicol Pathol* 1998; **11**: 127~131)

Key words: Sprague-Dawley rat, Lewis rat, retinal dysplasia, retinal degeneration, N-methyl-N-nitrosourea, apoptosis.

Introduction

N-methyl-N-nitrosourea (MNU), one of the alkylating agents distributed widely in the environment, has a specific target on photoreceptor cells, and has been shown to cause retinal degeneration when administered to a variety of animals in their young adult life¹⁻⁷. The retinal degeneration caused by MNU, which is similar to human retinitis pigmentosa^{8,9}, is characterized by the loss of photoreceptor cells by an apoptotic mechanism terminating ≤ 7 days after a single MNU administration³⁻⁷. In mice, MNU administered transplacentally at day 16 of gestation¹⁰ or intraperitoneally (ip) at day 0 or 3 of the neonatal period¹¹ has led to the development of retinal dysplasia. Retinal dysplasia is characterized histologically by dysplastic rosettes composed of variable numbers of retinal cells¹².

The age of the animal at which a chemical such as MNU is administered is critical in relation to the retinal cell response; the same chemical evokes different retinal lesions when administered at different ages. However, no study has been performed to examine the effect of MNU on the retinal response in rats at different ages. We therefore conducted a detailed study to determine the retinal lesions evoked by MNU in newborn (day 0), weanling (day 21), young adult (day 50), and aged (day 150) rats and to identify the MNU dose necessary to develop such lesions.

Materials and Methods

Animals

Sprague-Dawley (SD) and Lewis (LEW) rats were purchased from Clea Japan (Osaka), and Charles River Japan (Kyoto), respectively. A total of 166 (approximately half female and half male) rats were used. The animals were maintained on a basal diet, CMF (Orient Yeast, Kyoto) and water freely. The rats were housed in plastic cages with wood-chip bedding, in an air-conditioned room, at $22 \pm 2^\circ\text{C}$, relative humidity $60 \pm 10\%$, with a 12-hr light-dark cycle. The illumination intensity was < 60 lux at the cage level.

Chemical

N-methyl-N-nitrosourea (MNU) was obtained from Nacalai Tesque, Kyoto. On arrival, MNU was kept in -20°C in the dark. MNU solution was freshly prepared, dissolved in physiologic saline containing 0.05% acetic acid, just before use.

Experimental procedure

At 0 (day of birth), 21, 50, and 150 days of age, male and female SD and LEW rats received a single ip injection of 35, 50, 75, 90 and/or 105 mg/kg body weight of MNU. At 7 days after the treatment, rats were anesthetized by ether and killed by cervical dislocation. Some rats were killed 21 days after the treatment, and 4 SD and 4 LEW rats treated with physiologic saline containing 0.05% acetic acid at 0, 21, 50, and 150 days of age, respectively, and killed 7 days later were served as controls. After the animals were killed, both eyes were quickly removed, and complete autopsies were done on all animals. During the course of the experiment, animals were handled according to the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987).

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Tissue fixation and processing

In all cases, one eye was fixed overnight in 10% neutral buffered formalin; the other was fixed for 12 hrs in methacarn. The formalin- and methacarn-fixed tissues were routinely processed for paraffin embedding and stained with hematoxylin and eosin (H & E). All other macroscopically abnormal organs and tissues were dissected, fixed in 10% neutral buffered formalin, and processed for histological examination.

Immunohistochemistry

Serial sections were subjected to the immunohistochemical studies. Methacarn-fixed tissues were stained with anti-vimentin antibody (clone V9; Dako, Glostrup, Denmark) by a labeled streptavidin biotin kit (Dako, Carpinteria, CA) as described previously³. Antigen retrieval was performed for the demonstration of vimentin. In brief, the sections were placed in plastic staining jars containing citrate buffer solution (pH 6.0) and heated in a microwave oven for four 5 min cycles. After the jars were allowed to cool for 1 hr at room temperature, the sections were treated with anti-vimentin antibody (1:200 dilution) in a moist chamber at room temperature for 1 hr. The reaction products were visualized with DAB containing H₂O₂, and then the sections were counterstained with Bosma's hematoxylin.

Internucleosomal DNA fragmentation assay

Four 21-day-old LEW rats were administered an ip injection of 90 mg/kg MNU. Four other untreated age-matched LEW rats served as controls. Twelve hrs after the treatment, all eight animals were sacrificed, eyes were excised, and their corneas and lenses were removed. Genomic DNA was isolated from the eyes with the use of a Sepa Gene kit[®] (Sankyo, Tokyo) based on the guanidine thiocyanate extraction procedure as described previously¹³. An equal amount of each DNA sample (10 µg/lane) was subjected to electrophoresis on a horizontal 0.8% agarose gel containing ethidium bromide and then visualized under ultraviolet illumination. The presence of 180 bp ladder formation was determined.

Statistical analysis

The body weight changes of the MNU-treated rats among the different MNU dose groups were compared to those of the untreated rats at each time point and analyzed using Welch's *t* test.

Results

General remarks

Seven days after the treatment with MNU, the body weight changes of the treated rats were compared with those of the untreated controls. The changes were similar regardless of gender and the strain of rat. Therefore, the combined data with no regard to the strain and gender are presented. In day 0 MNU-treated rats, 105 mg/kg MNU caused a significant ($p < 0.01$) retardation of body weight gain; comparative body weight increase compared with the controls was 65%. In day 21, 50, and 150 MNU-treated rats, ≥ 50 mg/kg, ≥ 75 mg/kg, and ≥ 75 mg/kg MNU treatment, respectively, resulted in significant ($p < 0.01$, respectively) less weight gain compared to the controls, but the comparative

body weight increase compared with the controls was $> 82\%$. No moribund animals were seen within 7 days after MNU treatment in each age group. However, ≥ 90 mg/kg MNU treatment at day 0 caused death in all rats, and treatment at day 21 resulted in death in approximately half of the rats between 8–21 days after the treatment. MNU caused no deaths in the 50 and 150 day-old-treated rats during the 21-day observation period.

Retinal dysplasia in newborn rats

In the SD and LEW rats exposed to 50, 75, 90, or 105 mg/kg MNU at the day of birth and examined 7 days later, although no obvious change in the size of the eyeball was seen, regardless of strain difference and gender difference, a marked effect on the development of bilateral retinal dysplasia characterized by retinal rosettes was seen. Histologically, the abnormalities in the retina were the characteristic rosettes in the outer neuroblastic layer over the continuous lining single layer of pigment epithelial cells (Fig. 1). In some rats sacrificed at day 21, rosettes were located in the outer nuclear layer (Fig. 2). Immunohistochemically, haphazardly oriented vimentin-positive Müller cell processes

Fig. 1. Retinal dysplasia in an LEW rat exposed to 90 mg/kg MNU at the day of birth and examined 7 days later. Note the retinal rosettes in the neuroblastic layer. HE $\times 200$

Fig. 2. Retinal dysplasia in LEW rat exposed to 90 mg/kg MNU at the day of birth and examined 21 days later. Note the retinal rosettes in the outer nuclear layer. HE $\times 200$

Fig. 3. Retinal dysplasia in an LEW rat exposed to 90 mg/kg MNU at the day of birth and examined 7 days later. Haphazardly oriented vimentin-positive Müller cell processes are seen. Vimentin $\times 400$

were conspicuous (Fig. 3). MNU at a dose ≥ 50 mg/kg produced retinal dysplasia in almost all rats (**Table 1**). However, the severity of the disease (number of rosettes per rat) was not dose-dependent; a higher dose of MNU did not always result in a higher number of rosettes per eye.

Retinal response in weanling, young adult, and aged rats

Regarding the retinal response of SD and LEW rats exposed to MNU at day 21 (weanling), day 50 (young adult), and day 150 (aged), all retinal lesions, if present, were progressive retinal degeneration; degenerative photoreceptor cells were seen, and the outer nuclear layer was reduced to few layers of cells 7 days after MNU exposure (Fig. 4). In a few of the rats sacrificed at 21 days after treatment, the outer nuclear layer was lost, which resulted in inner nuclear cells in direct contact with the remnant pigment epithelial cells or with Bruch's membrane (Fig. 5). During the course of the degeneration, vertically oriented vimentin-positive Müller cell processes were evident (Fig. 6). Internucleosomal DNA fragmentation was seen in MNU-treated rats (12 hrs after the treatment), but not in age-matched control rats (Fig. 7). Thus, the photoreceptor degeneration was due to apoptosis.

Fig. 4. Retinal degeneration in an SD rat exposed to 35 mg/kg MNU at day 150 and examined 7 days later. Note the degenerative photoreceptor cells. HE $\times 200$

Fig. 5. Retinal degeneration in an LEW rat exposed to 90 mg/kg MNU at day 21 and examined 21 days later. Note the complete lack of photoreceptor cells. HE $\times 200$

Regarding the age of the animals, the MNU dose sufficient to consistently induce retinal degeneration in all treated rats was different across the age groups (**Table 1**). The MNU dose necessary to induce 100% retinal dysplasia at day 21 treatment was > 90 mg/kg, that at day 50 treatment was > 50 mg/kg, and that at day 150 treatment was ≥ 35 mg/kg. The age may affect the dose of MNU for the induction of retinal degeneration. The sensitivity of photoreceptor cells for the

Table 1. Age-specific and Dose-dependent Occurrence of Retinal Dysplasia and Degeneration in Sprague-Dawley (SD) and Lewis (LEW) Rats One Week after an Intraperitoneal Administration of N-methyl-N-nitrosourea (MNU)

Retinal response	Retinal dysplasia		Retinal degeneration					
	Age (day)							
MNU	0		21		50		150	
Dose (mg/kg)	SD	LEW	SD	LEW	SD	LEW	SD	LEW
35	ND	ND	ND	ND	0/4	3/4	3/3	3/3
50	7/7	4/4	0/7	0/4	1/4	4/4	3/3	3/3
75	7/7	4/4	4/7	0/4	4/4	4/4	3/3	3/3
90	2/7	4/4	6/7	4/4	ND	ND	ND	ND
105	7/7	4/4	7/7	ND	ND	ND	ND	ND

ND: not determined.

Fig. 6. Retinal degeneration in an LEW rat exposed to 90 mg/kg MNU at day 21 and examined 7 days later. Vertically oriented vimentin-positive Müller cell processes are evident. Vimentin $\times 400$

Fig. 7. DNA fragmentation assay. Internucleosomal DNA fragmentation is seen in the retina 12 hrs after 90 mg/kg MNU treatment to LEW rats (lane 2), but not in the age-matched control retinas (lane 1).

development of retinal degeneration increased in parallel with the age of the rats. In all age groups, the MNU treatment produced no significant alteration of ganglion cells or inner nuclear cells. No extraocular lesions were evident microscopically. The control retinas (both SD and LEW) examined at 157 days of age showed no abnormality.

Discussion

The ip inoculation of MNU to newborn (at day 0) SD and LEW rats resulted in bilateral retinal dysplasia characterized by the formation of rosettes, whereas the MNU treatment of weanling (at day 21), young adult (at day 50), and aged (at day 150) rats resulted in retinal degeneration characterized by photoreceptor cell loss. Regardless of gender and the strain of rat, different retinal lesions were evoked when MNU was administered at different time points. Moreover, the MNU dose necessary to induce retinal degeneration decreased with the rats' aging.

Among the newborn rats treated with 50 mg/kg MNU or more, retinal dysplasia was induced in almost all treated rats; the severity of the lesion (mean numbers of rosettes per rat) varied among the rats and was not dose-dependent. Retinal dysplasia can be experimentally induced in a variety of animals by infectious agents^{14,15}, by x-ray irradiation¹⁶⁻¹⁹, and by chemicals^{20,21}. Retinal rosettes can be induced transplacentally in mice inoculated with MNU on day 16 of gestation¹⁰, or by an ip inoculation at day ≤ 3 of the neonatal period¹¹. In agreement with these findings in mice, retinal dysplasia was successfully induced by a single ip inoculation of MNU to day 0 rats. The etiology of retinal dysplasia in humans, whether due to a genetic mechanism^{22,23}, an environmental disturbance²⁴, or a combination of the two factors is unknown. Although the factor(s) responsible for the occurrence of the dysplasia is not yet known, it is related to the stage of maturation of the retina^{11,17}; the immaturity is essential for the development of the disease²⁵. Human retinal dysplasia is a developmental abnormality present at birth; the maturity of the rat retina at birth is about equivalent to that of the retinas of 4- to 5-month-old human fetuses²⁶. The induction of retinal dysplasia in neonatal rats is thus not surprising. In radiation-induced retinal dysplasia in dogs¹⁷ and MNU-induced retinal dysplasia in mice¹¹, the lesions were eventually repaired. Therefore, long-term observations of the consequence of the lesions in the present rat model are necessary to determine whether the changes are irreversible. Retinal dysplasia may occur when pigment epithelium is lacking²⁵ or there is a defect in the development of Müller cells²⁷. In the present model, however, continuous retinal pigment epithelial cells were seen above Bruch's membrane, and a separation of the retina from the underlying pigment epithelium was not seen; abnormally oriented Müller cell processes was characteristic. The manifestation of rosettes may be the result of incomplete repair due to imbalance of abnormal cell death and cell proliferation caused by MNU¹¹.

Retinal degeneration was evoked in all treated rats when > 90 mg/kg, > 50 mg/kg and ≥ 35 mg/kg of MNU were administered to 21, 50, and 150-day-old rats, respectively; the older rat retinas were more sensitive to MNU. Older animals are more sensitive to mutagenic challenge caused by MNU and less able to repair the genetic damage²⁸. Since MNU does not require metabolic activation²⁹, the susceptibility to MNU appears to be primarily due to changes occurring within the photoreceptor cells. The sensitivity of photoreceptor cells to MNU is an age-dependent phenomenon. The causes of retinal degeneration in humans are multiple genetic mutations^{8,30}, and several animal models that carry mutant genes are known to develop retinal degeneration^{31,32}. After the weaning stage, when the rat retinal cells are morphologically and functionally fully developed²⁶, the

sensitivity of the photoreceptor cells to MNU differed in relation to age, and retinal degeneration characterized by the loss of photoreceptor cells by an apoptotic mechanism was confirmed by internucleosomal DNA fragmentation. Therefore, the MNU-induced retinal degeneration phenotypically reproduces human retinitis pigmentosa. Retinitis pigmentosa is a progressive disease whose onset is in early adult life^{8,9}.

In conclusion, two human eye diseases (retinal dysplasia and retinal degeneration) thought to be caused by different mechanisms were reproduced in rats by one chemical agent (MNU); the MNU treatment of actively growing retinas induced retinal dysplasia, and the MNU treatment of mature retinas resulted in retinal degeneration. The aged retinas were much more susceptible to MNU.

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