Effects of Gender and Gonad Status on N-Methyl-N-nitrosourea-induced Cataractogenesis and Retinopathy in Lewis Rats

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Abstract. Background: The effects of differences in gender and gonadal status on the occurrence of N-methyl-N-nitrosourea (MNU)-induced cataract and retinopathy were studied using ovary-intact, ovariectomized, testis-intact and testectomized Lewis rats. Materials and Methods: Castration was performed at 36 days of age, 50 mg/kg MNU was administered intraperitoneally at 50 days of age, and lens and retinal changes were evaluated at 260 days of age (210 days after MNU injection). Results: Although there was little difference in the incidence of cataract and retinopathy among the groups, ovary-intact rats had a significantly higher cataract index and retinal damage ratio (both are indicators of disease severity) than ovariectomized rats and testis-intact rats, respectively. However, the cataract index and retinal damage ratio did not correlate with the serum 17β-estradiol and progesterone levels, respectively. Conclusion: The presence of ovaries and female gender appear to be associated with greater severity of cataracts and retinopathy, respectively, but the severity of these diseases did not correlate with the serum hormone levels.

Cataract, an opacity of the lens that can cause blindness, is one of the most prevalent eye diseases and constitutes a significant health problem. Epidemiological evidence suggests that there is little difference in the incidence of cataract between men and women before the female menopause (1), but that an increase in the incidence of cataract in women coincides with the estrogen deficiency after menopause (1, 2). Delayed menopause appears to protect against cataract (3), as does hormone replacement therapy (3, 4). There is little difference in risk of cataract between estrogen-only and estrogen-progesterone hormone replacement therapy, suggesting that estrogen is involved in cataract etiology (5). This is consistent with the finding that long-term administration of the antiestrogen tamoxifen citrate is associated with increased risk of cataract (6), while evidence from laboratory studies also indicates that estrogen is involved in cataract etiology. In a study in which cataracts were induced by TGF-β in cultured rat lenses, the lenses from ovariectomized rats were more susceptible to cataract than those from ovary-intact rats, and that 17β-estradiol inhibited cataractogenesis in lenses from ovariectomized rats; progesterone did not counteract the cataract-inducing effect of TGF-β (7). In a study of N-methyl-N-nitrosourea (MNU)-induced cataractogenesis, administration of estrogen to ovariectomized rats reduced the incidence of MNU-induced cataract; ovary-intact rats were not included for comparison (8).

Retinitis pigmentosa is an eye disease characterized by loss of photoreceptor cells leading to visual disturbance (9). Estrogen has been found to have neurotrophic and neuroprotective properties (10, 11). Studies show that estrogen protects against damage to retinal neurons in vitro (12), and against light-induced (12) and ischemia/reperfusion-induced damage to retinal neurons in vivo (13). Although epidemiological evidence does not indicate gender differences in the incidence of retinitis pigmentosa in humans, systemic administration of 17β-estradiol significantly protects against light-induced photoreceptor cell loss in ovariectomized rats (12), whereas progesterone has no such effect (12, 14). Although the mechanisms underlying the protective effects of estrogen are unclear, it appears that estrogen affects cells in various parts of the eye. However, the available evidence does not clearly indicate whether pathologies of the lens and photoreceptor cells are associated with gender or gonadal status (endogenous hormone levels).

Several experimental models have been developed for studies into the occurrence of eye disorders. When administered to rats, MNU damages lens epithelial cells.
and photoreceptor cells, causing cataract and retinopathy, respectively (15-17). Prepubescent rats are more susceptible to MNU-induced cataractogenesis than adult rats (15, 16), whereas adult rats develop cataracts 6 to 8 months after a single systemic administration of MNU (18-20). In adult rats, 60 to 75 mg/kg MNU induces retinopathy and photoreceptor cell loss over a 7-day course, whereas the retinopathic effects of 50 mg/kg MNU take significantly longer to become detectable (17, 21). There is no gender difference in the development of cataract or retinopathy in prepubescent rats (15, 16, 22). Studies indicate that female rat lenses and photoreceptor cells are less susceptible to MNU-induced damage than those of males (18). However, there have been no precise studies of the effects of gender or gonadal status on occurrence of MNU-induced cataract or retinopathy in rats of reproductive age. Therefore, the aim of the present study was to elucidate the cellular responses underlying the effects of differences in gender and gonadal status on occurrence of MNU-induced cataract and retinopathy in adult ovary-intact, ovariectomized, testis-intact and testectomized Lewis rats. Changes in the lens and retina were evaluated 30 weeks after administration of MNU.

Materials and Methods

Animals. Pregnant Lewis rats were obtained from Charles River Japan (Hino, Japan), and their pups were born in our animal facility. Those pups (male and female) were used in the present experiments. The rats were housed in plastic cages with wood-chip bedding in an air-conditioned room at 22±2°C and relative humidity of 60±10%, with a 12-h light/dark cycle. The illumination intensity was below 60 lux at the cage level. The rats were fed a commercial pellet diet (CMF; Oriental Yeast, Chiba, Japan) and water ad libitum throughout the experiment. All procedures performed on experimental animals were approved by the Animal Experimentation Committee of Kansai Medical University, Japan.

Experimental procedures. At 36 days of age (before puberty), approximately half of the females and males were castrated, and the gonads of the other half were left intact. In Lewis rats, vaginal opening occurs from 40 to 43 days of age (average, 40.9±0.4 days). At 50 days of age, all animals received a single intraperitoneal injection of 50 mg/kg MNU. The MNU was purchased from Nacalai Tesque (Kyoto, Japan), stored at −20°C in the dark and dissolved in physiological saline containing 0.05% acetic acid immediately before the injection. After the injection of MNU, ovari-intact rats developed mammary tumors. Mammectomy was immediately before the injection. After the injection of MNU, ovari-intact rats developed mammary tumors. Mammectomy was immediately performed when the largest mammary tumor reached a diameter of ≥1 cm. The animals were weighed once per week, and were killed 210 days after MNU treatment; only rats that survived until 210 days after MNU treatment were used in the remaining procedures and analysis. Randomly-selected rats (6 rats per group) were killed by ether anesthesia and their blood was collected via a cardiac puncture. The sera were analyzed for 17β-estradiol and progesterone, using respective radioimmunoassay kits (Diagnostic Products, Los Angeles, CA, USA). All animals were autopsied and both eyes and any abnormal organs and tissues were processed for histological examination.

Tissue processing. Both eyes were removed from each rat; one eye from each pair was fixed in methacarn, and the other eye was fixed in 10% neutral buffered formalin. Methacarn- and formalin-fixed eyes were embedded in paraffin wax, and were then sectioned at a thickness of 4 μm through the center of the eyeball, parallel to the optic axis and nerve (including the ora serrata and optic nerve). The sections were stained with hematoxylin and eosin (HE). Also, tissue samples were obtained from all the MNU-induced tumors and were examined histologically.

Immunohistochemistry. Serial sections of paraffin-embedded tissues were examined by immunohistochemistry and TUNEL staining, using a procedure described elsewhere (23). Briefly, methacarn-fixed sections were stained with anti-α-smooth muscle actin antibody (clone 1A4, DakoCytomation, Glostrup, Denmark), anti-vimentin antibody (clone V9, DakoCytomation), or anti-glial fibrillary acidic protein antibody (GFAP; clone 6E2, DakoCytomation), using a labelled streptavidin biotin kit (DakoCytomation, Carpinteria, CA, USA). TUNEL staining was performed using the formalin-fixed sections and an apoptosis detection kit (Apop-Tag, Intergen, Purchase, NY, USA). Positive staining was visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemicals, Osaka, Japan) as the chromogen.

Cataract index. The degree of lens abnormality was evaluated histologically using the cataract index and a method described elsewhere (15). Briefly, to grade the cataracts in routinely-prepared methacarn-fixed and HE-stained sections, the severity of 7 features were evaluated: i) lens epithelial apoptosis, ii) lens epithelial desquamation, iii) multilayered spindle epithelium, iv) lens fiber swelling, v) liquefaction/vacuolar change, vi) calcification and vii) bizarre nuclei of lens fiber cells. These features were arbitrarily scored as follows: absent (grade 0), slight (grade 1), moderate (grade 2), severe (grade 3), or very severe (grade 4). The cataract index was defined as the sum of each score.

Retinal damage ratio. To evaluate the retinal damage ratio, color images of methacarn-fixed and HE-stained sections were obtained as JPEG files using a Nikon ECLIPSE E100M camera and Nikon Act-1 software version 2.62. The image files were analyzed using Lumina version 1.10β 1 (Mitani, Tokyo). Damage to the retina was defined as the presence of less than 4 rows of photoreceptor nuclei across the width of the retina (24). The retinal damage ratio was defined as follows: (damaged retinal length/whole retinal length) x100. MNU-induced retinal damage starts at the central retina and progresses to the peripheral retina (25).

Statistical analysis. All discrete values were expressed as mean±SE. Differences in incidence of cataract and retinopathy were analyzed using the Chi-square test. Differences in body weight, serum hormone levels, cataract index and retinal damage ratio were first analyzed for homogeneity of variance, and were then evaluated using the Kruskal-Wallis non-parametric test or non-repeated measures ANOVA parametric test. For differences with a prespecified p value of <0.05, a posthoc test (Bonferroni/Dunn's procedure) was performed and p values of <0.05 were considered to indicate significance.
Results

General findings. At 30 weeks after MNU injection, 74 rats had survived (15 ovary-intact, 17 ovariectomized, 24 testis-intact and 18 testectomized). All the surviving rats were in good overall health and were, therefore, considered suitable for tissue sampling and analysis. Body weight gain occurred in all 4 groups (Figure 1); testis-intact rats were heaviest, and ovary-intact rats were lightest ($p<0.01$). Body weight was significantly increased by ovariectomy, compared with ovary-intact rats ($p<0.01$). Body weight was not significantly reduced by testectomy, compared with testis-intact rats. There was no significant difference in body weight between castrated males and females. Ovary-intact rats developed multiple mammary tumors synchronously and metachronously. Starting 13 weeks after MNU injection, mammectomy was performed when the largest tumor in a rat reached a diameter of $\geq 1$ cm. A total of 68 mammary tumors developed (4.5 mammary tumors per rat), and all were histologically confirmed to be mammary adenocarcinomas. In addition to mammary carcinomas, 2 testis-intact rats developed Zymbal gland tumors just before they were killed, and their tumors were histologically confirmed to be squamous cell carcinomas. The data describing the occurrence of carcinomas is summarized in Table I. No tumors or abnormalities other than carcinomas and eye lesions were observed. Serum 17β-estradiol levels were high in ovary-intact rats, and were significantly lower in testectomized rats; ovariectomized and testis-intact rats had high levels (Table II). Serum progesterone levels were high in ovary-intact rats, and were significantly lower in ovariectomized and testis-intact rats.

Cataract. Ovary-intact rats were the first group to develop lens opacities, 19 weeks after MNU injection. The degree of lens opacity varied widely, with some rat lenses appearing clear under macroscopic inspection, but showing early histological signs of cataractogenesis. Histological examination of clear or slightly opaque lenses revealed that the opacities were due mainly to damage to the cortex. In lenses that appeared clear under gross examination, the nucleated fibers in the bow area of the equatorial region showed swelling and had a granular appearance indicating early cataractogenesis. In macroscopically visible cataracts, histological examination revealed calcified degenerative fibers, but the lesions remained in the cortex. Immunohistochemistry showed a lack of $\alpha$-smooth muscle actin deposition in the damaged lenses, while TUNEL
signals were not detected in damaged lenses (data not shown). All groups had comparable (high) levels of incidence of histologically-confirmed cataract, whereas the cataract index differed considerably among the groups (Table III). The cataract index was significantly higher for ovary-intact rats than for ovariectomized rats ($p<0.01$).

However, the cataract index did not correlate with serum hormone levels.

Retinopathy. Retinopathy was observed in all rats (Table IV). Some retinas were totally damaged, whereas others less so. The degree of retinopathy did not correlate with the severity of cataract. In all damaged retinas, the central area around the optic nerve was severely damaged, but the peripheral area was intact. In the injured part of the retina, loss not only of photoreceptor cells, but also of inner retinal cells was observed (Figure 2a); lost photoreceptor cells and inner retinal cells were replaced by vimetin- and GFAP-positive cells (Figure 2b). In the uninjured peripheral retina (Figure 2c), vertically oriented Müller cell processes were vimetin, and GFAP, positive (Figure 2d). Retinal damage ratios indicated that the retinas of ovary-intact rats were significantly more severely damaged than those of testis-intact rats ($p<0.05$). However, the retinal damage ratio did not correlate with the serum hormone levels.

Discussion

Different strains of rats exhibit different relationships between gender and occurrence of retinopathy. For example, the incidence of spontaneous retinopathy in Sprague-Dawley rats is twice as high for females as for males (25). In Fisher 344 rats, spontaneous retinopathy develops more rapidly and is less severe in females than in males (26). MNU-induced retinopathy in Wistar rats develops more slowly in females than in males (18). In the present study, at 30 weeks after MNU injection, the
severity of retinopathy was greater for females than for males. In a previous study, the degree of photoreceptor cell damage caused by continuous exposure to light was significantly less for rats ovariectomized before puberty (≤5 weeks of age) than for ovary-intact rats (27). In another study, administration of 17β-estradiol to rats ovariectomized after puberty significantly protected them against light-induced retinopathy and loss of photoreceptor cells (12). The effects of estrogen are biphasic, and it appears that estrogen can increase or decrease the loss of photoreceptor cells, depending on the dose (28). The timing of ovariectomy and the dose of MNU may influence the effects of estrogen on retinal photoreceptor cells. In the present study, ovariectomy was performed before puberty (vagina was not opened), and the ovariectomized rats tended to have less severe retinopathy than ovary-intact rats. Although gender and gonadal status appear to affect the severity of retinopathy, the relationships between the serum hormone levels and severity of retinopathy are unclear. Interestingly, in the present study, 30 weeks after MNU injection, all 4 groups of rats exhibited not only loss of photoreceptor cells, but also loss of inner retinal cells. In another study, 20 weeks after Sprague-Dawley rats had been injected with MNU, photoreceptor cell loss was the only observed change, inner retinal elements remaining intact (21). Thus, the retinal response after injection of MNU can differ between different strains of rats. Similarly, in human retinitis pigmentosa, degeneration of inner retinal cells occurs secondary to degeneration of photoreceptor cells (29).

In Wistar-derived Alderley Park rats, cataracts develop spontaneously, with a incidence for females (14%) which is higher than for males (4%); cataracts have not been observed in castrated males or females less than 2 years of age (30). In BALB/c mice homozygous for the Nakano cataract gene (nct/nct), cataract develops sooner in females than in males; for mice of reproductive age, ovariectomy plus testosterone treatment retarded the onset of cataract, compared with ovary-intact and ovariectomized (without testosterone treatment) rats (31). The presence of endogenous estrogen may accelerate the development of cataracts in mice of reproductive age. In the present study, the cataracts of ovary-intact females were significantly more severe than those of ovariectomized rats, and tended to be more severe than those of males. Thus, the presence of ovaries appears to increase the susceptibility to MNU-induced cataractogenesis in reproductive-age Lewis rats. However, the testectomized rats were not more resistant to cataractogenesis than the other groups, and cataractogenesis did not correlate with the levels of serum 17β-estradiol or progesterone. In other studies, MNU-induced cataracts in Wistar rats were less severe in females than in males (18), and administration of estrogen to Sprague-Dawley rats ovariectomized at 7 weeks of age (after onset of puberty) reduced the incidence of MNU-induced cataract, compared to ovariectomized rats without estrogen (8). These differences in the effects of MNU may be due to the timing of ovariectomy (before or after puberty) or differences between rat strains.

In conclusion, we found that gender and gonadal status affected retinopathy and cataractogenesis in Lewis rats after injection of MNU. Further study is needed to evaluate the relationships between endogeneous hormones and the progression of these diseases.

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