Corneal Damage Induced in Adult Mice by a Single Intraperitoneal Injection of N-Ethyl-N-Nitrosourea

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Abstract. Aim: The morphological response and cell kinetics of the mouse cornea to various doses of N-ethyl-N-nitrosourea (ENU) was examined. Materials and Methods: ENU at a dose of 50, 100, 200, 400, or 600 mg/kg was injected intraperitoneally into female BALB/c mice at seven weeks of age. Sequential morphological features and cell kinetics (TUNEL assay as apoptosis marker, PCNA immunostaining as proliferative activity marker, and p63 immunostaining as corneal stem cell marker) of corneal damage caused by 600 mg/kg of ENU were also analyzed 6, 12, 24 and 72 h, and 7 days after exposure. Moreover, older mice (25 to 34 weeks of age) received the same dosage and were sacrificed 7 days later. Both eyes of all mice were analyzed histopathologically and morphometrically, by using the parameters of corneal epithelial thickness. Results: All ENU-treated mice in the 600 mg/kg group developed corneal damage characterized by desquamation and loss of epithelial cells within 7 days. Corneal epithelial thickness was significantly reduced in the 600 mg/kg group as compared to the control group and decreased to approximately half of the normal thickness. Although the number of TUNEL-positive epithelial cells in the ENU-treated mice was similar to that of the control mice, ENU inhibited the proliferative activity of epithelial cells showing PCNA-positivity 72 h after treatment. The p63-positivity of epithelial cells decreased in the central cornea of mice treated with 600 mg/kg of ENU. Older mice did not develop corneal damage from exposure to ENU. Conclusion: ENU induced corneal damage in adult mice, and epithelial cell loss was caused by the inhibition of corneal epithelial proliferation. This is the first report to describe ENU-induced corneal injury in adult mice.

Most medications have side-effects on various body tissues and functions, including the eye. Organ toxicity is readily detectable in the eye, although the beneficial effects of therapy are also readily seen (1). The human cornea can be damaged by systemic exposure to drugs and chemicals, including anticancer drugs. DNA-alkylating agents induce keratitis (busulfan, chlorambucil, cyclophosphamide, cytosine arabinoside, deoxycoformycin, 5-fluorouracil (5-FU), corneal opacity (carmustine, tamoxifen), and corneal perforation or ulceration (mitomycin C, erlotinib) (2-7). Injuries to the outer surface of the cornea are caused by chemical irritation, chemical accumulation, induction of cell death, and/or inhibition of cell proliferation and differentiation, via tear fluid that gets into the eye (8, 9). In some cases, high-dose chemotherapy can promote hypolacrimation, followed by keratoconjunctivitis sicca, which is commonly referred to as dry eye syndrome (3).

N-ethyl-N-nitrosourea (ENU) is an alkylating agent that is particularly effective in inducing numerous types of tumors in rodents, including tumors of the nervous tissue, gastrointestinal tract, pancreas, respiratory tract, skin, mammary gland, kidney, and lymphoreticular tissues (10, 11). ENU mutagenesis has also been widely used to create a large number of random point mutations in genomic DNA (12), and it is a powerful tool for creating disease models (13), such as those for eye morphological defects, corneal opacity, and reduced corneal thickness (14-16). ENU also induces progressive retinal degeneration via retinal photoreceptor cell apoptosis in mice (17). In contrast, few reports that focus on ENU-induced corneal damage have been published. The present study focuses on the effect of ENU on corneal epithelial cells in adult BALB/c mice.
Materials and Methods

Animals. Fifty 6-week-old female SPF/VAF mice [BALB/c AnNCrlCrlj] were purchased from Charles River Japan (Osaka). After their arrival, mice were maintained in specific pathogen-free conditions and fed a commercial diet (CMF; Oriental Yeast, Chiba, Japan) with free access to food and water. Animals were housed in plastic cages with paper-chip bedding (Paper Clean, SLC, Hamamatsu, Japan) in an air-conditioned room at 22±2°C and 60±10% relative humidity with a 12-h light/dark cycle. The illumination intensity was below 60 lux in the cages. Corneal mineralization occurs spontaneously in this strain of mice (18, 19); however, mineralization was not detected histopathologically in any of the mice used in the present study. All procedures were in accordance with the Guidelines for Animal Experimentation of Kansai Medical University.

Chemical and dose formulation. ENU (ISOPAC®; chemical formula, C3H7N3O2) was obtained from Sigma-Aldrich (Tokyo, Japan) and was kept at –80°C in the dark. The ENU solution was dissolved in physiological saline just before use.

Experimental procedure. At 7 weeks of age, ENU at a dose of 50, 100, 200, 400, or 600 mg/kg or vehicle (physiological saline) was administered by an intraperitoneal (i.p.) injection. Each dosage group contained 5 mice. All mice were sacrificed 7 days after ENU or vehicle treatment under anesthesia by using Forane® (isoflurane; Abbot Japan, Tokyo, Japan). In another experiment, 20 mice received a single i.p. injection of 600 mg/kg of ENU and 4 randomly selected mice were sacrificed at 5 time points (6, 12, 24, and 72 h, and 7 days) after treatment. To examine the susceptibility to ENU in relationship to aging, six female mice at 25 and 34 weeks of age received an i.p. injection of 600 mg/kg ENU or vehicle and were sacrificed seven days after treatment. All mice were observed daily for clinical signs of toxicity and were weighed at the time of ENU injection and on the day of sacrifice. At the time of sacrifice, both eyes were quickly removed, and complete necropsies were conducted on all animals.

Tissue fixation and processing. One eye was fixed in 10% neutral buffered formalin, and the other was fixed in Methacarn (60% methanol, 30% chloroform and 10% acetic acid) overnight, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (HE). To confirm the degree of immunosuppressant effect and hematopoiesis, the thymus and spleen were fixed in 10% neutral buffered formalin and tissue sections were used to prepare HE-stained slides.

TUNEL, PCNA, and p63 immunostaining. Formalin-fixed eye sections at 5 time points (6, 12, 24, and 72 h, and 7 days) after ENU treatment and at 7 days after vehicle (physiological saline) treatment were used for cell kinetic analysis of ENU-induced corneal damage. Cell death was observed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP digoxigenin nick end-labeling (TUNEL) by using an in situ apoptosis detection kit (Apop-Tag; Millipore, Billerica, MA, USA) according to a previous report (17). Sequential sections were immunohistochemically evaluated with anti-proliferating cell nuclear antigen (PCNA) monoclonal antibody (clone PC10, 1:100 in dilution; Leica Biosystems, Newcastle upon Tyne, UK), a proliferative activity immunomarker (20), and anti-p63 monoclonal antibody (clone 4A4, 1:100 in dilution; Thermo Fisher Scientific, Fremont, CA, USA), a corneal stem cell immunomarker (21-24). A labeled streptavidin-biotin (LSAB) staining kit (Dako, Carpinteria, CA, USA) and antigen retrieval by pressure-cooker heating (Pascal, Dako) were used for immunostaining.

Morphometric analysis of corneal epithelial thickness. HE-stained sections of the eyes were scanned to make digital images by a high-resolution digital slide scanner (NanoZoomer 2.0 Digital Pathology; Hamamatsu Photonics, Hamamatsu, Japan). The ndpi image files were opened in color mode by specific viewer software, NDP.view (Hamamatsu Photonics). Epithelial thickness of the central cornea was individually measured from Methacarn-fixed slides by using NDP.view. Clinical, histopathological and morphometrical evaluations were performed by a toxicologic pathologist certificated by the Japanese Society of Toxicologic Pathology (K.Y.) and an ophthalmologist certificated by the Japanese Ophthalmological Society (K.M.).

Statistical analysis. All discrete values, expressed as the mean±standard error (SE) were analyzed by using the two-tailed independent Student’s t-test for unpaired samples after confirming the homogeneity of variances. The results presented below include comparisons between the ENU-treated mice and the vehicle-treated mice. P-values of <0.05 were considered to show significant differences.

Results

General remarks. Acute death within 5 days occurred in two out of five mice treated with 600 mg/kg ENU. In the same group, all mice had closed eyes from the first day of treatment. Body weight gain was affected in a dosedependent manner, however, ENU did not cause the loss of body weight (data not shown). The older mice (25 and 34 weeks old) treated with ENU displayed a decrease in body weight gain without having closed eyes. None of these mice died during the experimental period.

Morphological and morphometric analysis of dose-dependent corneal damage. Corneal histology on ENU-treated mice was compared with that of saline-treated mice 7 days after treatment. In saline-treated mice, epithelial cells in the central cornea composed about 6 layers of cells: basal cells (one cell layer), wing cells (three to four cell layers), and superficial cells (one to two cell layers) (Figure 1a). In contrast, 600 mg/kg ENU caused the corneal epithelia to be reduced to two to three layers of cells at the central cornea of all mice (Figure 1b). Corneal epithelia were severely desquamated. The remaining epithelia consisted of one cell layer each of basal cells, wing cells, and/or superficial cells. Basal cells contained vacuolated cytoplasm, indicating edematous change (Figure 1b). In one out of five 600 mg/kg ENU-treated mice, inflammatory cell infiltration was present in the anterior chamber. The peripheral cornea did not exhibit histopathological changes in any of the cell layers. Moreover, in the groups treated with 400 mg/kg ENU and less, all corneas were intact (Figure 1c-f). In the corneal stroma of all...
mice, the clefts within the collagenous stroma represented artifacts of tissue processing. Histopathologically, ENU did not cause any changes to the corneal stroma or endothelial cells. Vascular proliferation, fibrosis, and mineralization were not detected in the corneas of any mice examined in this study.

Seven days after 600 mg/kg ENU treatment, the corneal epithelial thickness significantly decreased in the central cornea, as compared with that of the saline-treated controls (Figure 2). In mice treated with 400 mg/kg or less of ENU, the epithelial thickness at the central cornea was not significantly different. Compared to 37.3 μm as the mean thickness of the central epithelia in the saline-treated group, the mean thicknesses was 21.0 μm in the 600 mg/kg group, 36.6 μm in the 400 mg/kg group, 37.1 μm in the 200 mg/kg group, 36.9 μm in the 100 mg/kg group, and 37.5 μm in the 50 mg/kg group.

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Figure 1. Corneal changes in adult mice 7 days after a single i.p. injection of ENU. a: Saline control, b: 600 mg/kg, c: 400 mg/kg, d: 200 mg/kg, e: 100 mg/kg, f: 50 mg/kg. In saline-treated mice, corneal epithelium consisted of ca. six cell layers: basal cells (one cell layer), wing cells (three to four cell layers), and superficial cells (one to two cell layers) (a). In contrast, corneal epithelium of mice treated with 600 mg/kg ENU were severely desquamated and composed only two or three cell layers (b). Basal cells showing vacuolated cytoplasm, indicating edematous change. No corneal epithelial changes were seen in mice treated with 400 mg/kg or less of ENU (c, d, e, f). The clefts within the collagenous stroma represent artifacts of tissue processing. HE staining, Methacarn fixation, ×400.

Figure 2. Corneal epithelial thickness 7 days after a single i.p. injection of ENU. Mice treated with 600 mg/kg ENU had a statistically significant decrease in the thickness of their central corneal epithelial layer, as compared to saline-treated controls. **p<0.01.
In the other organs examined, the most striking features were the dose-dependent suppression of hematopoiesis and atrophy in the thymus and spleen in the mice that received 400 mg/kg or more of ENU (data not shown). Thus, the cause of acute death in the highest dose group was hematopoietic toxicity and immunotoxicity, as previously reported (17).
Morphological and morphometric analysis of sequential changes in corneal damage. Compared with the control retina (Figure 3a), no distinct changes were seen 6 or 12 h after 600 mg/kg ENU treatment (Figure 3b and c). The first evidence of corneal change in the central area was desquamation and flattening of superficial cells that occurred after 24 h (Figure 3d). The desquamation progressed to the loss of corneal epithelia at 72 h (Figure 3e). Finally, the corneal epithelial layer decreased to two to three cell layers 7 days after treatment (Figure 3f). No bacterial or viral infections were seen in any of the mice. Morphometrically, compared with the normal control corneas, the thickness of corneal epithelia in the central corneas of ENU-treated mice decreased progressively (Figure 4). The thickness of the corneal epithelia was 35.9 μm, 35.4 μm, 33.3 μm, 30.5 μm, and 21.0 μm at 6, 12, 24 and 72 h, and 7 days, respectively.

Aging effect on ENU-induced corneal damage. A dose of 600 mg/kg ENU did not induce corneal damage in the central corneal area of 25- to 34-week-old mice, as their corneas appeared similar to those of the control corneas (Figure 5a and b).

TUNEL, PCNA, and p63 immunostaining. In both the control and 600 mg/kg ENU-treated mice, TUNEL signals appeared only in some superficial cells of all corneal epithelial areas, and thus an increased number of positive cells was not detected at any time points after ENU treatment (data not shown). No TUNEL-positive basal cells were detected in any of the treated mice. Many basal and wing cells were positive for PCNA antibody in the control corneas (Figure 6a); however, few or no signals were seen in corneas 72 h and 7 days after ENU treatment (Figure 6b). Most basal and wing cells in control corneas were positive for p63 antibody.
(Figure 6c); in contrast, these signals were seen only in some basal cells of ENU-treated cornea (Figure 6d). TUNEL, PCNA, and p63 signals were not detected in corneal stromal cells and endothelial cells of control and 600 mg/kg ENU-treated mice.

Discussion

The present study provides new evidence of corneal damage in response to ENU. Corneal damage characterized by the desquamation and loss of the epithelial layer in the central cornea was detected seven days after 7-week-old mice received a single i.p. injection of 600 mg/kg ENU. In older mice treated with 600 mg/kg ENU, corneal epithelial loss was not seen. Thus, the age at exposure influenced the ENU-induced corneal damage in adult mice.

The corneal epithelium undergoes dynamic turnover due to the sustained proliferation of basal epithelial cells. These basal cells are then displaced outward by the next generation of mitotic cells, and eventually they are lost by desquamation (23, 25). A slow centripetal movement of peripheral epithelial cells also contributes to the renewal of the epithelium; cells move centripetally from the limbus to the central cornea during a period of 8-9 weeks in mice (23). Once an epithelial cell is displaced from the basal layer, approximately one week is required for it to reach the corneal surface and desquamate (26, 27). The mechanisms that regulate corneal proliferation under normal physiological conditions and in disease states are multifactorial and complex.

In the present study, the corneal damage caused by 600 mg/kg of ENU was characterized by desquamation and cell layer loss and the disappearance of PCNA positivity of corneal epithelial cells. However, ENU did not induce increased cell death of corneal epithelial cells, as determined by TUNEL positivity. In contrast, p63-positive cells were located in basal cells and wing cells of the control cornea, in agreement with a previous report (22), while the p63-positivity of corneal epithelial cells was decreased in 600 mg/kg ENU-treated mice. This protein has been proposed to be a marker of keratinocyte stem cells in the skin and cornea (23, 24). Thus, ENU might affect the inhibition of epithelial cell proliferation, especially of corneal stem cells, and cause the progressive desquamation of the remaining epithelial cells. The cornea eventually reaches only two or three cell layers, consisting of basal cells, wing cells, and/or flattened superficial cells.

These corneal changes may result in reduced visual acuity and ocular irritation (9). The cornea has an exquisite sensitivity, as the epithelium is richly innervated by sensory nerve endings. Damage to the corneal epithelium is therefore very painful, with the level of discomfort varying with the degree of damage (2). All mice treated with 600 mg/kg ENU had closed eyes from the first day of treatment, suggesting eye pain due to ENU-induced corneal damage.

Systemic medications and chemical exposures reach the cornea via tear film, aqueous humor, and the limbal vasculature, and these substances may induce corneal damage including phospholipidosis (vortex keratinopathy), degeneration of basal epithelial cells with formation of epithelial microcysts, or stromal or endothelial deposition (2, 9). Antimetabolites, such as 5-FU and cytosine arabinoside (Ara-C), have antiproliferative properties that have been described in a tissue culture model of rabbit corneal epithelial cells (28). After treatment for five days, corneal epithelial cell proliferation was inhibited to 50% of control proliferation by 0.6 mg/l 5-FU or 30 mg/l Ara-C. 5-FU dramatically reduces the mitotic rate of corneal epithelia in rabbits in vivo, which is consistent with the known mechanisms of action of 5-FU in inhibiting DNA synthesis (8). Ara C induces corneal damage in humans (6) by causing the migration of the necrotic basal cells to the wing cell layer and finally to superficial epithelial layers. Moreover, high-dose chemotherapy can promote or increase the severity of hypolacrimation, followed by keratoconjunctivitis sicca, which is known as dry eye syndrome (3). Damage to the corneal epithelium occurs by alterations in the tear film secondary to cytotoxic damage to the lacrimal glands and/or the mucosa-producing goblet cells of the conjunctiva. Next, direct toxicity to the corneal surface by exposure to substances causes damage to the surface epithelium (29). In the present study, we did not histopathologically examine the lacrimal gland or conjunctiva, and the effect of ENU on tear flow or quality was not evident.

Alkylating agents induce DNA adduct formation of target cells, followed by cell death or gene mutation (30). N-Methyl-N-nitrosourea (MNU), an alkylating agent similar to ENU, causes DNA adduct formation in photoreceptor nuclei, followed by down-regulation of Bcl-2, up-regulation of Bax, and activation of caspase families, followed by retinal degeneration (31, 32). In these MNU models, no corneal changes, including adduct formation, have previously been reported. Further investigation of the difference in sensitivity would be necessary to understand the detailed pathogenesis of alkylating agent-induced corneal damage.

In conclusion, adult mice that received a single injection of ENU developed corneal damage due to the inhibition of corneal epithelial cell proliferation. Detailed mechanistic investigations of ENU-induced corneal lesions are needed for a better understanding of the pathogenesis of corneal damage induced by alkylating agents in humans.

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