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Structural, Morphological, and Functional Correlates of Corneal Endothelial Toxicity Following Corneal Exposure to Sulfur Mustard Vapor

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PURPOSE.
Sulfur mustard (SM) is a highly reactive vesicant that causes severe ocular injuries. Following exposure to moderate or high doses, a subset of victims develops a chronic injury known as mustard gas keratopathy (MGK) involving a keratitis of unknown etiopathogenesis with secondary keratopathies such as persistent epithelial lesions, corneal neovascularization, and progressive corneal degeneration. This study was designed to determine whether SM exposure evokes acute endothelial toxicity and to determine whether endothelial pathologies were specifically observed in MGK corneas as opposed to healed corneas.

METHODS.
Corneas of New Zealand white rabbits were exposed to SM vapor, and the corneal endothelium was evaluated at 1 day and 8 weeks using scanning electron microscopy (SEM), transmission electron microscopy (TEM), in vivo confocal microscopy (IVM), and fluorescent microscopy. Barrier function was measured by uptake of a fluorescent dye injected into the anterior chamber.

RESULTS.
A centripetal endothelial injury at 1 day was observed by SEM, TEM, IVM, and fluorescent microscopy. In vivo confocal microscopy revealed additional cytotoxicity between 1 and 13 days. In contrast to healed corneas, which appeared similar to sham-exposed naive eyes at 8 weeks, MGK corneas exhibited significant evidence of continued pathological changes in the endothelium.

CONCLUSIONS.
Endothelial toxicity occurs at the right time and with the appropriate pathophysiology to contribute to MGK. Based on these findings, we propose a model that explains the relationships among SM dose, the biphasic progression, and the various clinical trajectories of corneal SM injury and that provides a mechanism for temporal variations in MGK onset. Finally, we discuss the implications for the management of SM casualties.

Keywords: sulfur mustard, corneal endothelial cells, mustard gas, mustard gas keratopathy, corneal edema
reaching baseline levels by 6 weeks. In contrast, MGK corneas develop a persistent edema as soon as 3 weeks with recurring epithelial toxicity, basement membrane zone degeneration, and redundant deposition of basement membrane components following cyclical attempts to regenerate the epithelium. Secondary pathologies such as epithelial bullae, neovascularization, and limbal stem cell niche dysfunction subsequently appear, further interfering with stable repair of the ocular surface. The distinct pathophysiology of MGK revealed in these studies suggests the involvement of injury mechanisms that operate on different time scales and in different corneal compartments than during the acute injury. However, these findings did not reveal a pathology that was expressed in a spatiotemporal fashion to be responsible for the pathogenesis of MGK.

The first clinical symptom of MGK onset is a sustained edema, raising the possibility that chronic edema is associated with the pathogenesis of MGK. In rabbits, edema is localized in the posterior central cornea at 2 weeks, immediately before MGK onset. Given the anisotropic hydrodynamics of the stroma, this suggests that the origin of edema is also centrally located. Because (1) the corneal epithelium regenerates an intact epithelial cap by 5 days after exposure that remains intact until after MGK onset and (2) neovascularization does not extend to the central cornea until approximately 8 weeks, the most likely source of corneal edema is dysfunction of the corneal endothelium. Hypothesizing that the acute or chronic SM injury may involve endothelial toxicity, we evaluated the structure and function of the corneal endothelium during the acute SM injury and in resolved versus MGK corneas at 8 weeks.

METHODS

Ethics Statement and Disclaimers

The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense (United States Department of Agriculture certificate No. 51-F-0006). All procedures were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were conducted in accord with the principles stated in the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (Pub L No. 89-544), as amended.

Animals

Sixty female New Zealand white rabbits (Charles River Laboratories, Germantown, MD) weighing 2.0 to 2.5 kg were housed individually. Rabbits were provided a standard diet with regular enrichment and water ad libitum. At either 1 day or 8 weeks after SM exposure, rabbits were anesthetized with an intramuscular administration of 15 mg/kg of ketamine and 7 mg/kg of xylazine and euthanized by cardiac injection of sodium pentobarbital. Globes were enucleated and corneas processed as described below.

Exposure Procedures

Rabbits were exposed in cohorts of 8 to 16 animals during a 4-month period. One day before exposure, a 4-in² region on each rabbit’s back was clipped, and a fentanyl patch (25 µg/h) was placed anterior to the scapula. On the day of exposure, rabbits were anesthetized with an intramuscular administration of 15 mg/kg of ketamine and 7 mg/kg of xylazine, and physiological parameters were recorded. The right corneas of anesthetized rabbits were exposed to SM vapor for 2.5 min using a vapor cup delivery system as previously described. Two minutes after exposure, exposed eyes were gently rinsed with 10 mL sterile saline to flush residual agent. Rabbits were returned to cages and provided food and water ad libitum. Fentanyl patches were replaced after 72 hours to manage discomfort through 6 days after the exposure and applied liberally thereafter as needed. Animals were monitored daily for signs of pain and distress. Corneal injury was clinically evaluated on a regular basis using pachymetry, fluorescein exclusion assays, and slitlamp evaluations.

![Figure 1](image_url). Representative ×70 SEM demonstrating the centripetal nature of the endothelial SM injury, with a central denuded region and increased CEC retention at the injury penumbra. Scale bar: 100 µm.
Whole-Mount Fluorescent Microscopy

The cornea and lens (n = 6 each, sham and exposed) were excised from the globe using iridectomy scissors and immersion fixed for 15 minutes in 4% paraformaldehyde (Sigma-Aldrich Corp., St. Louis, MO) in PBS. Fixed corneas were washed thoroughly in PBS with 0.1% saponin (PBSS; Sigma-Aldrich Corp.) and then blocked overnight at 4°C in PBSS plus 3% BSA (Sigma-Aldrich Corp.) in a 12-well dish. Corneas were incubated with 330 nM phalloidin–Alexa 555 (Invitrogen, Carlsbad, CA) in PBSS for 20 minutes, washed three times with PBSS, and mounted on a slide with coverslip using Prolong DAPI Gold (Invitrogen). After drying, corneas were imaged using a Zeiss LSM 700 confocal microscope and analyzed with Zen 2009 (Carl Zeiss Microscopy, LLC, Thornwood, NY).

Electron Microscopy

Following enucleation, buffered 2.5% glutaraldehyde was injected into the aqueous humor via the cornea at the limbal margin. A venting needle was inserted into the cornea directly opposite the injection site to alleviate injection pressure. Globes were then immersed in 2.5% glutaraldehyde for 1 day. Corneal caps were excised using iridectomy scissors, postfixed in buffered 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in Poly/Bed 812 embedding resin (Polysciences, Inc., Warrington, PA) for transmission electron microscopy (TEM) analysis. Ninety-nanometer-thick sections were mounted on copper mesh grids and counterstained with uranyl acetate and lead citrate. Corneal sections were imaged using a JEM-1230 transmission electron microscope (JEOL Ltd., Tokyo, Japan). For scanning electron microscopy (SEM), corneas were dissected from the globe using iridectomy scissors, and the central cornea was removed using an 8-mm biopsy punch. Tissue punches were postfixed in buffered 1% osmium tetroxide, dehydrated in graded ethanol, and critical point dried. Mounted samples were ion beam coated using gold/palladium and imaged using a 7401F field emission scanning electron microscope (JEOL Ltd.). Observations of sequelae in at least 75% of corneas were considered to be genuine. Measurements of corneal size were performed by measuring the longest axis of 150 cells per cornea, three corneas per condition.

Measurements of Endothelial Permeability

Rabbits were euthanized 24 hours after exposure. Five minutes after euthanasia, 20 μL of a 0.1 mg/mL solution of AlexaFluor 488 (Life Technologies, Carlsbad, CA) dissolved in PBS (pH 7.4) was injected into the anterior chamber through a 30-gauge needle using a 100-μL Hamilton glass syringe (Hamilton Company, Reno, NV). The sham-exposed contralateral eye was injected first, followed by the exposed eye. After 10 minutes, corneas were excised and washed three times with PBSS, and mounted on a slide with coverslip using Prolong DAPI Gold (Invitrogen). After drying, corneas were imaged using a Zeiss LSM 700 confocal microscope and analyzed with Zen 2009 (Carl Zeiss Microscopy, LLC, Thornwood, NY).
Corneal Exposure to Sulfur Mustard Vapor

**RESULTS**

**SM Vapor Exposure Causes Corneal Endothelial Cell Vesication Within 24 Hours**

Corneas visualized at ×70 by SEM 24 hours after SM exposure exhibited a centripetal injury, with extensive loss of corneal endothelial cells (CECs) in the central cornea and increased retention toward the exposure margins (Fig. 1). The diameter of the acellular central lesion varied from 0.5 to 1 mm, with an additional 1-mm to 2-mm lesion periphery characterized by the patchy retention of cells. The effect of vapor SM exposure on endothelial cell loss was evaluated at higher resolution using fluorescent microscopy imaging of F-actin distribution. In sham-exposed corneas, a regular pattern of polygonal cells was observed, with predominantly hexagonal shape and peripheral accumulation of actin (Figs. 2A, 2D). Following SM exposure, corneas exhibited a spectrum of injuries ranging from gross endothelial sloughing to scattered loss of CECs (Figs. 2B, 2C). In areas with significant cell loss, the remaining CECs were often highly disorganized and enlarged (Figs. 2E, 2F). Nuclear staining indicated the presence of nuclear fragmentation and swelling, characteristic of terminally injured CECs (Figs. 2G–I).

**SM Exposure Results in Increased Endothelial Permeability In Vivo at 24 Hours**

The ability of the endothelium to maintain corneal deturgescence depends on osmotic pump activity and the integrity of the CEC monolayer. To determine whether SM exposure results in the acute disruption of endothelial function, stromal absorption of a fluorescent dye injected into the aqueous humor was quantified 1 day after exposure. The SM-exposed corneas absorbed 20-fold more fluorophore than control corneas in a 10-minute period (n = 8, P < 0.001) (Fig. 3), confirming that SM exposure increased endothelial permeability.

**SM Induces Changes in Endothelial Structure and Morphology at 24 Hours**

To obtain a more comprehensive overview of SM-induced changes in the corneal endothelium, the fine structure of the posterior cornea was evaluated by electron microscopy. En face scanning electron micrographs of sham-exposed corneas revealed a continuous layer of polygonal cells of regular shape and size, with interdigitated borders, apical microvilli, and infrequent cilia (Fig. 4A). Within 24 hours of exposure, all corneal endothelia exhibited evidence of an acute lesion, with extensive central CEC loss and more diffuse vesication in the exposure penumbra. The CECs within the exposed region displayed two general morphologies, namely, enlarged (highly attenuated) polymorphic cells and rounded or spindle-shaped cells (Fig. 4B). Most CECs exhibited atypical apical membrane morphologies and lacked cell-to-cell interdigitations (Figs. 4B, 4C). In regions of CEC vesication, denuded Descemet’s membrane (DM) was covered by a complex arbor of CEC lamellipodia and filopodia (Fig. 4D). The TEM imaging of corneal cross-sections confirmed the centripetal injury pattern, with CEC morphology progressively normalizing toward the injury margin (Fig. 5). Denuded DM near the central lesion was infiltrated by extensively arborized cellular processes. At more distal regions, overlapping cellular processes with loss of junctional complexes was common, suggestive of a motile process.
population. The rounded CEC population observed by SEM was found exclusively overlying polymorphic endothelium and displayed signs of necrosis or apoptosis.

**Endothelial Disruption Is Quantitatively Characterized**

In vivo confocal microscopy is a noninvasive technique that allows evaluation of CEC morphology in situ in a narrow region of the cornea. It was used to verify that postmortem evidence of endothelial injury at 24 hours was not an artifact of corneal processing. Before exposure, endothelia displayed a characteristic morphology with a regular mosaic appearance, cell density of 3219 cells/mm², and predominantly hexagonal shape ($n = 26$) (Fig. 6A). The 18 corneas that could be successfully imaged by IVM 24 hours after exposure exhibited indications of endothelial injury, with varied morphologies (Figs. 6B, 6C). In 39% (7 of 18), clusters of CECs were separated by regions of denuded DM. The remaining corneas (61% [11 of 18]) exhibited a more diffuse distribution of cell loss. Because IVM images a narrow region of the corneal endothelium, we anticipate that the variability is a consequence of whether images were captured nearer to the center or periphery of the SM lesion. Based on SEM findings, the corneas that could not be imaged may have had complete loss of endothelium in the region under examination, which would prevent IVM. Attempts to further quantify the area of CEC loss proved to be impossible due to the small field of view relative to the lesion size, limiting 1-day findings to gross descriptions of injury pattern.

The same rabbits were evaluated by IVM at 13 days to determine whether there was additional CEC loss (Figs. 6D, 6E). Among the 46% (12 of 26) of rabbits that were successfully imaged at both time points, CEC density was decreased by 29% at 24 hours ($P < 0.001$) and an additional 21% by 13 days ($P < 0.01$) (Fig. 6F). At 13 days, the CECs appeared more evenly distributed, and individual cell size seemed to have increased compared with controls, suggesting that the endothelial wound-healing response was in progress. Notably, the one resolving cornea in this cohort exhibited the most normal appearance at 13 days (Fig. 6D).

**MGK and Resolved Corneas Exhibit Distinct Endothelial Morphologies**

Endothelial cell morphology and structure were compared between MGK ($n = 24$) and resolved ($n = 6$) eyes 8 weeks after exposure. Resolved eyes were distinguished by the absence of

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**Figure 4.** Scanning electron micrographs of the apical surface of the corneal endothelium following corneal exposure to SM vapor. (A) Sham-exposed control demonstrating an intact endothelial monolayer with frequent microvilli and interdigitations at regions of cell–cell contact. (B–D) Characteristic morphologies observed 1 day after SM exposure. The inset in (D) is a higher-magnification view of the filopodia spreading over denuded DM. Scale bars: 10 μm, except for the inset, where scale bar: 1 μm.
Corneal Exposure to Sulfur Mustard Vapor

DISCUSSION

Involvement of Endothelial Toxicity in Acute and Chronic Corneal SM Injuries

Previous work has shown that SM vapor applied to the surface of the cornea penetrates to the anterior chamber within minutes, suggesting that the potential exists for endothelial toxicity. Changes in corneal endothelial morphology of rabbits following SM exposure have been described using specular microscopy, and evidence of endothelial injury has been identified in human MGK corneas, including reduced CEC density with increased variability in size and morphology. Although these findings suggest that SM injury may involve an endothelial component, the role of CEC toxicity in the pathophysiology of acute or chronic corneal SM injuries has not been evaluated to date.

Using multiple imaging methods, we found that corneal exposure to SM vapor results in an acute CEC toxicity with a centripetal injury pattern characterized by severe CEC loss at the lesion center and more diffuse CEC loss toward the injury margins. The existence of this central lesion in vivo was corroborated by IVM, and the effect of this lesion on endothelial integrity was functionally confirmed by an in vivo endothelial permeation assay. The IVM further indicated that CEC loss and endothelial disorganization persisted between 1 and 13 days, providing a potential mechanism for sustained edema beyond 2 weeks despite regeneration of an impermeant epithelial cap. Although IVM offers the powerful capability of evaluating longitudinal changes in individual corneas, imaging is compromised in conditions of edema, ocular haze, and complete endothelial loss. The inability to image half of the exposed corneas at 13 days suggests that our findings may be biased in favor of the lesser-injured corneas. Regardless, the successful demonstration of confocal imaging indicates that IVM will be a valuable technique to evaluate longitudinal changes in SM-injured corneas.

Unlike the resolved corneas, which were structurally similar to sham-exposed eyes, the MGK corneas exhibited an idiosyncratic endothelial injury characterized by focal CEC loss, abnormal CEC morphologies, and a diffusively thickened DM. The appearance of corneal fibrosis suggests the endothelial-to-mesenchymal transition of corneal CECs, a pathological response to severe endothelial injury that involves fibroblastic transformation and deposition of atypical basement membrane components. While we cannot discount that the endothelial toxicity observed at 8 weeks is secondary to anterior corneal degeneration, persistent epithelial lesions such as those observed during MGK generally do not have a significant effect on endothelial integrity, particularly during such a short period. Moreover, epithelial bullae, which are characteristic of severe endothelial decompensation, are an early clinical indicator of MGK onset in the vapor cup model, suggesting that anterior keratopathies are a consequence of endothelial failure. Alternative mechanisms for the chronic endothelial injury include DNA:SM adducts that exert delayed genotoxic effects or a subpopulation of CECs with slowly developing cytotoxic responses. We are currently characterizing longitu-
dinal changes in endothelial morphology to better understand the nature of this delayed CEC toxicity.

**Implications of Endothelial Injury to the Etiopathogenesis of MGK**

In the human corneal endothelium, gaps due to CEC loss are exclusively filled by spreading of proximal CECs. These morphological changes compensate for endothelial loss until the barrier between the cornea and aqueous humor can no longer be maintained, resulting in persistent corneal edema and secondary anterior keratopathies. Because adult human CECs do not proliferate in vivo, any loss of CECs therefore represents a permanent reduction in endothelial capacity. However, as in humans, sufficiently severe injury to the rabbit endothelium also results in irreversible corneal decompensation and secondary keratopathies.

Based on these findings, we hypothesize that SM-induced endothelial failure may be the causal mechanism underlying MGK pathogenesis. This hypothesis is consistent with the dose dependence between SM and the development of MGK that has been observed in humans and rabbits, as well as the different clinical trajectories (resolved chronic MGK and delayed-onset MGK) that have been reported in human casualties. According to this hypothesis, corneal exposure to low doses of SM results in an acute epithelial lesion, with minimal endothelial toxicity, and corneas recover without long-term complications. Alternatively, exposure to doses of SM that cause irreparable injury to the corneal endothelium would result in endothelial barrier failure, producing a persistent edema with secondary anterior keratopathies. Following a severe injury, there may be no apparent delay between the acute injury and MGK onset,

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**Figure 6.** Representative IVM images of a cornea before (A) and after (B–E) SM exposure. (A) Baseline image. (B, C) Characteristic appearance 1 day after SM exposure. Note the different appearances of the endothelium. In (C), hyperreflective nuclei presumably result from the prominent nuclear morphology observed in Figure 4. (D, E) Endothelial morphology in corneas that would go on to resolve (D) or develop MGK (E). Scale bars: 100 μm. (F) Longitudinal changes in the average endothelial cell density (±SD) in corneas before and after exposure (n ≥ 11 at each time point). *P < 0.05; ***P < 0.001.)
resulting in the chronic clinical form of MGK. The more common delayed-onset form of MGK might be a hybrid of these two outcomes, such that endothelial barrier function is initially restored, but the subsequent loss of CECs due to age, corneal injury, and/or delayed cytotoxicity reduces CEC density below the compensation threshold, resulting in persistent edema and MGK.26,27 Determining the severity of endothelial injury that would be sufficient to evoke MGK in either humans or rabbits is not straightforward, in part because nonpenetrating, focal lesions in the corneal endothelium are an unusual injury modality. While charts of compensation threshold can be used to estimate the effect of cell density on the function of human endothelia, these charts have been developed from the aging-related loss of CECs and thus may not be relevant to injuries involving the focal loss of large numbers of CECs.28 Evidence of such endothelial lesions in this study broaches the question of how the distribution of CEC loss can affect the ability of the endothelium to recover. For example, in the case of an evenly distributed loss of CECs, morphological changes in the surviving cells might rapidly allow the restoration of endothelial barrier function. In contrast, if the loss of a similar number of CECs occurred as a focal lesion, recovery may be delayed by the need for centripetal migration of surviving CECs from the lesion periphery. Consequently, in cases of sufficiently large focal lesions, the endothelial barrier may not be able to recover before the onset of irreversible secondary pathologies.

Clinical Implications of SM-Induced Corneal Endothelial Toxicity

The specific association of endothelial injury and dysfunction with MGK in both humans and rabbits has led us to hypothesize that SM-induced endothelial lesions that exceed the repair capacity of the endothelium manifest as MGK. This emphasis on endothelial injury as the etiological basis of MGK explicates the shared symptoms of the chronic and delayed-onset forms of MGK despite the different clinical time frames, provides a mechanism for sustained corneal edema despite regeneration of an impermeable corneal epithelial cap by 5 days after exposure in rabbits, and suggests that the timing of MGK onset is a function of acute CEC cytotoxicity and the subsequent rate of loss. It is also consistent with the observation that other clinical endotheliopathies involving the rapid loss of large quantities of CECs such as aphakic bullous keratopathy exhibit symptoms that are similar to MGK, including epithelial bullae, delayed limbal stem cell deficiency, and corneal inflammation.14,29–32 For these conditions, the only effective treatment is currently corneal transplant surgery.

**FIGURE 7.** Scanning micrographs showing CEC morphologies in resolved (A) and MGK (B–D) corneas 8 weeks after SM exposure. The inset in (A) is a sham-exposed cornea at the same magnification, highlighting the increased size of CECs in resolved corneas. Note the different morphologies in (B–D) as described in the text, suggesting ongoing cytotoxicity and a delayed healing response. Scale bars: 10 μm.
To date, there has been little effort to characterize endothelial function in human MGK victims. Although analysis has been severely limited by the poor structural integrity of MGK corneas, histology and IVM have indicated an endothelial component to the SM injury. Establishing a causal relationship between corneal pathologies and SM exposure is further complicated by the fact that human casualties are decades beyond the point of exposure. Nonetheless, the possibility of an endothelial contribution to corneal pathogenesis has important implications for SM therapeutic research. First, the failure to address an endothelial injury may explain why candidate therapies targeting the anterior cornea have had limited efficacy in preventing MGK. Second, if the extent of endothelial injury correlates with the likelihood of MGK onset, then quantitation of endothelial toxicity (e.g., by IVM) may offer a method to distinguish between eyes that will resolve and those that are at risk of developing MGK. Third, we hypothesize that early clinical interventions to restore endothelial function such as deep lamellar keratoplasty may mitigate or prevent the appearance of MGK. Given the ambiguities regarding the effect of endothelial cell proliferation on MGK onset and progression, longitudinal evaluations of CEC numbers, behavior, and morphology will be critical in studies that use the rabbit model to evaluate therapies to retain or restore the corneal endothelium. Fourth, if SM results in corneal endothelial injury, the possibility exists of toxicity to more posterior tissues at sufficiently high doses, including the retina.

CONCLUSIONS

In conclusion, we provide in vivo and postmortem evidence of corneal endothelial toxicity in acute and chronic ocular SM injuries. Based on these findings, we have synthesized a novel mechanistic explanation for the persistent edema observed in eyes that develop MGK. Endothelial injury provides a single mechanism to explicate the dose dependence of MGK onset (determined by permeation of cytotoxic quantities of SM to the endothelium), as well as the chronic and delayed-onset trajectories of MGK. This model proposes that the clinically biphasic injury progression is an epiphenomenon arising from the regenerative capacity of the corneal endothelium and hypothesizes that the probability and timing of MGK onset are functions of CEC loss. The endothelial injury suggests that therapies to restore the endothelium may mitigate the likelihood of MGK and that quantitation of endothelial loss may provide a diagnostic to identify corneas likely to develop MGK. We anticipate that these findings will motivate additional attempts to elucidate the putative pathogenic roles of endothelial injury in acute injuries and in SM survivors exhibiting chronic ocular sequelae.

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References
