Corneal Nerve Disruption Reactivates Virus in Rabbits Latently Infected with HSV-1

Craig F. Beyer, James M. Hill, James J. Reidy, and Roger W. Beuerman

Trauma, inflammation, and neuronal stimulation or damage can reactivate latent herpes simplex virus type 1 (HSV-1). The innervation density of the corneal epithelium is 300–600 times that of skin and, therefore, corneal nerve disruption could provide a strong stimulus for HSV-1 reactivation. This study has documented HSV-1 ocular reactivation following three methods of corneal nerve disruption in rabbits. Twenty HSV-1 latently infected rabbits (26 eyes) were divided into three groups: 7 rabbits received uniocular cryogenic injury, 7 rabbits underwent uniocular anterior superficial keratectomy, and 6 rabbits had binocular transection of the corneal nerves at the corneoscleral limbus which, in contrast to the other treatments, produced minimal epithelial change. Opposite eyes in the first two groups of rabbits were left undisturbed to serve as HSV-1 infected controls. Three additional rabbits, not infected with HSV-1, underwent gold chloride impregnation of the corneal nerves for light microscopic documentation of corneal nerve damage induced by each procedure. On all HSV-1 infected eyes, daily HSV-1 ocular cultures were obtained for 7 consecutive days. All three procedures resulted in marked corneal nerve destruction and degeneration. HSV-1 shedding occurred in 5/7 (71%) of the eyes that underwent cryogenic lesioning; in 5/7 (71%) of the eyes that underwent anterior keratectomy; and in 8/12 (67%) of the eyes that had the corneal nerves transected at the corneoscleral limbus. Only 4 (29%) of the 14 control eyes had positive HSV-1 ocular cultures. This investigation provides strong evidence that corneal nerve disruption is correlated with ocular HSV-1 reactivation.

The mechanism of herpes simplex virus type 1 (HSV-1) reactivation remains unclear even though current HSV-1 reactivation models have many features in common. In addition to reactivation, most methods involve some degree of corneal trauma, inflammation, neuronal stimulation, or damage to the nerves that innervate the cornea. Transcorneal iontophoresis of epinephrine, timolol, and 6-hydroxydopamine with dipivefrin hydrochloride induces HSV-1 shedding in the preocular tear film and results in HSV-1 recurrent corneal epithelial lesions in rabbits latently infected with HSV-1. Green et al. have shown that electric stimulation of the trigeminal ganglia induces ocular HSV-1 reactivation in rabbits. Nesburn et al. have shown that mechanical stimulation of the trigeminal ganglia induces HSV-1 ocular shedding in latently infected rabbits. Recently, Romanowski et al. have reported HSV-1 ocular shedding induced by an intrastromal injection of water. Finally, corneal incisions from radial keratotomy and penetrating keratoplasty result in HSV-1 ocular shedding and recurrent HSV-1 corneal epithelial lesions in rabbits latently infected with HSV-1. The most consistent feature common to all these methods of ocular HSV-1 reactivation appears to be neuronal stimulation or disruption of the nerves that innervate the cornea.

Intravenous injection of cyclophosphamide and dexamethasone has been shown to induce HSV-1 reactivation, and Haruta et al. were able to demonstrate recurrence of corneal epithelial lesions. Although immunosuppression appears to be the most likely mechanism of reactivation, Haruta et al. postulated that cyclophosphamide may be directly toxic to ganglionic cells, and thereby induce viral DNA replication. Therefore, this model may represent an additional form of neuronal alteration that induces HSV-1 recurrences.

The current study was undertaken to document HSV-1 recurrences following three methods of corneal nerve disruption: cryogenic injury of the cornea, anterior superficial keratectomy, and transection of the corneal nerves at the corneoscleral limbus. The first two procedures, freezing and mechanical injury, induce damage to the cornea requiring prolonged re-
rabbits. Corneal sections were placed in Tris-HCl chloride solution for 15 min. After blotting, the corneas were placed in acidulated water for 14–15 hr. Corneas were removed, blotted, and placed into 70% ethanol to stop the staining reaction. The corneal tissues were dehydrated with increasing ethanol concentration (80%, 90%, absolute) and finally a brief exposure to xylene. Tissues were mounted flat with Permount on a glass slide for light microscopic observation and photography.

Virus

HSV-1 McKrae strain was propagated on primary rabbit kidney (PRK) cell monolayers and titered by plaque assay on green monkey kidney cell (CV-1) monolayers. The virus was frozen in small aliquots at −70°C. The same batch was used for inoculation of all rabbits.

Rabbits and Viral Inoculation

Both unscarified corneas of New Zealand white rabbits (1.5–2.5 kg) were inoculated with a 20-μl suspension of HSV-1 McKrae strain (5 × 10^6 pfu/ml). Primary corneal infection (dendritic and geographic epithelial lesions) was verified by slit-lamp biomicroscopic examination (SLE) on postinoculation (PI) days 4–8. The care and maintenance of the rabbits used in these experiments conformed to the ARVO Resolution on the Use of Animals in Research.

Tear Film Swabs

Preocular tear film was collected from each rabbit eye on a sterile Dacron-tipped swab by gentle rotation of the swab in the upper cul-de-sac and then into the lower fornix, where the swab was allowed to absorb the tear film for 5 sec. To minimize the damage to the corneal epithelium, care was taken to avoid swabbing the cornea. The swabs were immediately placed in tubes containing tissue culture medium and confluent PRK cell monolayers, and incubated for 18–24 hr at 37°C in a CO₂ incubator. Subsequently, the swabs were squeezed against the side of the tubes to remove excess medium and removed. Eagle's minimum essential medium (KC Biological, Lenexa, KS) with 2% fetal bovine serum (1 ml) was added for nutrition and pH adjustment. The tubes were monitored daily for 7–9 days for the appearance of cytopathic effects indicative of HSV-1.

Slit-Lamp Biomicroscopic Examination

Only eyes that had transection of their corneal nerves underwent SLE with fluorescein staining, after swabbing to collect the tear film, to detect HSV-1 corneal epithelial lesions. The lesions were characterized as deep punctate lesions, dendritic lesions, or geographic epithelial defects. At the time of examination, the results of the eye swab data were not known to the observer. Therefore, all SLE data were obtained in a masked fashion with respect to eye swab results. Due to extensive epithelial disruption, no data are presented for SLE of eyes that underwent cryogenic injury and anterior superficial keratectomy.

Corneal Nerve Disruption

Prior to corneal nerve disruption, rabbits were anesthetized with an intramuscular injection of 1.0–1.5 ml of a 5:1 mixture of ketamine HCl (100 mg/ml) and xylazine HCl (100 mg/ml). Two to 3 drops of topical proparacaine were administered to both eyes. Cryogenic injury was produced by applying a circular (8–9-mm diameter) piece of dry ice against the surface of the cornea for exactly 30 sec. This formed an ice ball over the entire cornea that resolved within 1 min after the ice was withdrawn. Anterior superficial keratectomy was performed by partial trephination into the superficial corneal stroma with a 7.0-mm trephine. The edge of the trephinated area was grasped with forceps and peeled from the central surface of the cornea. Finally, transection of corneal nerves was performed under the operating microscope. Eight to 10 corneal nerves were identified as they entered clear cornea near the limbus where they were transected with the tip of a No. 11 Bard-Parker blade.
rior superficial keratectomy, and 6 rabbits (12 eyes) underwent transection of the corneal nerves at the corneoscleral limbus on postinoculation days 42–82. Opposite eyes in the first two groups of rabbits served as controls (14 eyes). Starting on the day of lesioning, daily ocular HSV-1 cultures were performed on all eyes for 7 days to detect viral shedding. Daily SLEs were performed only on eyes that underwent transection of the corneal nerves at the corneoscleral limbus. SLEs were not useful in the eyes that underwent cryogenic injury or anterior keratectomy because these procedures left large epithelial defects that precluded the differentiation of epithelial HSV-1 recurrences.

Results

The pattern of corneal nerve disruption and degeneration induced by each procedure was documented initially in uninfected rabbits to avoid the complicating factor of recurrent lesions (Figs. 1-3). The cryogenic lesion produced an inflammatory response within the corneal stroma, as well as neural degeneration. Within the zone that had been frozen, there was a loss of intraepithelial terminals. Deeply-lying neural structures showed ghosts of Schwann tubes; however, they were not disrupted by freezing. Anterior keratectomy results in an avulsion of the epithelium and of stromal layers containing the subepithelial plexus and deep stromal nerves. However, this procedure results in a dramatic increase in intraepithelial collateral sprouts growing to the wound margin. These results agree with previously published work.11

In contrast to the above procedures, transection of deep stromal nerve trunks at the limbus had no effect on the central corneal epithelium as judged by SLE. At 24 hr, the small incisions had healed, and the cornea was otherwise normal. Aesthiometry at this time showed a pattern of dense anesthesia and areas of minimal sensory loss. Light microscopic examination showed areas of epithelial devoid of nerve terminals. Regeneration of neural structures in the stroma could be followed to the transected nerve bundle (Figs. 3A, B). This procedure resulted in little or no inflammatory response.

The numbers of eyes in each group with positive HSV-1 ocular cultures are summarized in Table 1. In the eyes that underwent cryogenic injury, HSV-1 was detected in the preocular tear film in five (71%) of the seven eyes for a period of 1-4 days, and 15 (33%) of the 47 eye swabs in this group were positive for HSV-1. In the opposite eyes of these rabbits, only one (14%) of the seven control eyes and only 1 (2%) of the 49 ocular swabs were positive for HSV-1. Therefore, cryogenic injury significantly increased the number of HSV-1 positive cultures (P < 0.01).

As in the eyes that received cryogenic injury, five (71%) of the seven eyes that underwent anterior keratectomy had positive HSV-1 ocular cultures, and 15 (31%) of the 48 eye swabs in this group were positive for HSV-1. In the opposite control eyes, three (42%) of the seven eyes and 7 (14%) of the 49 ocular swabs were positive for HSV-1. The number of positive ocular cultures was significantly greater in the eyes that underwent anterior keratectomy compared to the control eyes (P < 0.05).

In the rabbits that underwent bilateral transection of the corneal nerves at the corneoscleral limbus, five (83%) of the six rabbits and 8 (67%) of the 12 eyes had positive ocular HSV-1 cultures. Of the 72 ocular cultures obtained, 21 (29%) were positive for HSV-1. Viral shedding began 2 days after lesioning and ranged from 1 to 4 days with an average duration of shedding of 2.6 days. In addition, SLEs revealed that five (83%) of the six rabbits and 7 (58%) of the 12 eyes developed corneal epithelial lesions suggestive of recurrent HSV-1. Of the 7 eyes with HSV-1 epithelial lesions, 2 (29%) had deep punctate lesions, 3 (43%) had dendrites, and 2 (29%) had geographic epithelial defects.

Discussion

The cornea is innervated principally by fibers whose cell bodies originate in the trigeminal ganglion, and by a much smaller number from the superior cervical ganglion. After acute HSV-1 keratitis in rabbits, both of these ganglia have been shown to harbor latent HSV-1.12 The large limbal nerve bundles can be observed in rabbits and human corneas by using the slit lamp or with an operating microscope and retroillumination. These bundles are usually visible beyond a point of bifurcation. After leaving the limbus, the nerves pass through the middle third of the stroma, branching anteriorly, and forming a dense subepithelial plexus. From this plexus, the axons penetrate the basal lamina and supply sensory endings to the corneal epithelium.13

Recurrent corneal HSV-1 infections could occur when infectious virus from the trigeminal ganglion reaches the cornea along the sensory neural route by anterograde axonal transport. Recently, Rivera et al14 provided evidence of anterograde, intraxonal transport of HSV-1 particles in the corneal nerves after virus reactivation by epinephrine iontophoresis. Reidy et al15 demonstrated reactivation and axonal transport of HSV-1 following selective sectioning of corneal nerves in rabbits. In both studies, unmyelinated corneal axons were found to contain nonenveloped virions by electron microscopy. These findings suggest that corneal HSV-1 recurrences result from reactivation of latent HSV-1 in the trigeminal ganglion. Evidence for HSV-1 latency in the corneas of...
Fig. 1. (A) Cryogenic injury, 96 hr after injury. Several polymorphonuclear inflammatory cells and fibroblasts are present (small black arrows). Among the faint background of polygonal basal epithelial cells (large open arrow), degeneration and reduction in the number of intraepithelial nerves is apparent (×63). (B) Cryogenic injury, 96 hr after injury. In this section, leashes of regenerating intraepithelial collateral nerves (arrow) can be seen traversing the area of previous intraepithelial nerve destruction (×63).
mice, rabbits, and humans has also been reported. However, there is little direct evidence that HSV-1 that is latent in the cornea plays a role in epithelial recurrences.

Studies have shown recurrent HSV-1 infections in humans following surgical trauma to the sensory neurons of the trigeminal ganglion. Carton and Kilbourne were the earliest to associate reactivation of latent herpes simplex with trigeminal sensory-root section, and others have since shown reactivation occurring after decompression of the trigeminal nerve root. Hakanson reported an 80% incidence of clinical herpes labialis after injecting glycerol into the trigeminal cistern of several patients to treat trigeminal neuralgia.

The cornea is a highly innervated tissue. The innervation density of corneal epithelium is 300–600 times that of skin and 20–40 times that of tooth pulp. The results of this study support the concept that corneal nerve disruption, similar to trigeminal sensory-root disruption, reacts latent HSV-1. As depicted by the gold chloride studies and light microscopy, all three methods of corneal nerve disruption, cryogenic injury, anterior keratectomy, and transection of the corneal nerves at the corneoscleral limbus, resulted in degeneration of corneal nerves. In the eyes receiving cryogenic injury, the gold chloride studies revealed the presence of many inflammatory cells, as well as damaged corneal nerves. However, in the gold chloride studies of the eyes that underwent transection of the limbal corneal nerves, an inflammatory response was not evident, but a similar percentage of ocular swabs were positive for reactivated HSV-1. Therefore, we feel that corneal nerve damage, and not inflammation, was the primary cause of HSV-1 reactivation in this experiment.

Recently, Rose et al demonstrated that full-thickness circular corneal trephination for penetrating keratoplasty with postoperative corticosteroids significantly increased the rate of HSV-1 shedding in HSV-1 latently infected rabbits. In addition, Haruta et al demonstrated that corneal incisions from radial keratotomy, also in HSV-1 latently infected rabbits, significantly increased the frequency of HSV-1 ocular shedding and recurrent HSV-1 corneal epithelial lesions. Corneal nerves are severed during penetrating keratoplasty.
Fig. 3. A. Limbal incision, 72 hr after surgery. Intraepithelial nerves are largely absent in the area of the basal polygonal cells of the epithelium (A). Beyond this area, a deeper section of the cornea can be seen demonstrating degenerated stromal nerves (arrow) (×63). (B) Limbal incision, 72 hr after surgery. The empty Schwann cell sheaths (arrow) of the deep stromal nerves are more clearly visualized under higher magnification (×100).
Corneal nerve disruption reactivates latent HSV-1

Table 1. Ocular HSV-1 reactivation and corneal epithelial lesions

<table>
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<tr>
<th>HSV-1 reactivation</th>
<th>Cryogenic injury</th>
<th>Anterior keratectomy</th>
<th>Transection of corneal nerves</th>
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<tr>
<td></td>
<td>Injured (%)</td>
<td>Control (%)</td>
<td>Injured (%)</td>
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<tr>
<td>No. of eyes with positive HSV-1</td>
<td>5/7 (33)</td>
<td>1/7 (14)</td>
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<tr>
<td>HSV-1 ocular cultures</td>
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<td>1/49 (14)</td>
<td>15/48 (71)</td>
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<td>(2)</td>
<td>(31) *</td>
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<tr>
<td>No. of eyes with HSV-1 lesions by SLE</td>
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For cryogenic injury and anterior keratectomy, controls were opposite eyes of same animal.

* Cryogenic lesioning and anterior keratectomy disrupted the corneal epithelium so that HSV-1 epithelial lesions could not be discerned by SLE.

Acknowledgments

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