Pathogenesis of corneal oedema associated with herpetic eye disease

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Abstract

Corneal oedema and stromal disease, induced in rabbits by intrastromal injection of herpes simplex virus, type 1, strain RE (HSV-1, RE), reached a peak of 12-15 days after infection. Corneal oedema as measured by ultrasonic pachymetry, and stromal disease as measured by a subjective scoring system, were closely related for 30 days after infection. Morphometric analysis of wide field specular micrographs showed that no immediate endothelial cell damage occurred in either control or HSV-1 infected corneas. Alizarin red S staining of corneas taken during the period of most severe oedema indicated no significant endothelial cell loss; however, visual inspection indicated numerous staining abnormalities. Scanning and transmission electron microscopy provided evidence of an intact endothelial layer possessing integrated infiltrating cells. Virus antigen could not be detected on endothelial cells by immunoperoxidase staining at any time during development of corneal oedema. The results indicate that corneal oedema associated with HSV-1 induced disease can occur in the absence of detectable virus replication and cytolysis of corneal endothelial cells.

Corneal oedema is associated with numerous manifestations of herpes simplex virus type 1 (HSV-1) induced ocular disease.¹ Oedema may develop with the progression of acute disease into the corneal stroma when infection is produced by strains of HSV-1 which have a strong tendency to produce stromal disease.² Disciform corneal oedema may follow acute corneal infection or appear during recrudescence in the presence or absence of obvious epithelial disease.³ Corneal oedema is often a significant feature of keratouveitis produced by HSV-1 infection.⁴

The oedema produced in association with these varying forms of HSV ocular disease may spontaneously resolve or persist and contribute to the development of chronic stromal disease and necrotising stromal keratitis.⁵ The pathophysiological mechanisms responsible for the corneal oedema associated with this spectrum of HSV induced diseases are unknown. However, one would anticipate that the corneal endothelium, as a major regulator of water balance in the cornea, would have a central role. Sundmacher⁶ has proposed that disciform oedema, for example, represents a viral disease of the endothelium involving replication of the virus in the endothelium followed by attack of immunocompetent cells on infected endothelial cells, the latter being more harmful to the endothelium.

However, little direct evidence exists to confirm the presence of virus or viral antigen in the endothelium during the course of disease.

Virus has been cultured from the anterior chamber of patients with endotheliitis, but the site of replication was not positively established.⁷ The literature contains other reports of endothelial defects including endothelial cell loss associated with herpes virus induced anterior segment disease.^{8,9} Clinically, however, it is often difficult to evaluate the condition of the endothelium during disease because in many cases corneal oedema interferes with slit-lamp examination and specular microscopy. In addition, the source of virus cannot be positively identified without enucleation and careful study of the ocular tissues.

Animal experiments have provided some important information on the state of the corneal endothelium in HSV induced disease, though the mechanisms responsible for corneal oedema have not been well characterised. The injection of live virus into the anterior chamber of rabbit eyes results in the apparent replication of virus in corneal endothelial cells and corneal oedema.^{10,11} Growth of virus has been observed in cultures of HSV infected rabbit corneal endothelial cells^{12,13} (O'Brien *et al*, unpublished data). Movement of virus to endothelial cells and replication in those cells may reflect both a strain dependent character of the virus and the immune status of the host. Spread of virus from an epithelial site of inoculation to the endothelium occurs with some strains of HSV-1, while other strains do not disseminate or require corticosteroids to promote spread of virus to the endothelium.¹¹ Irvine and Kimura¹⁴ demonstrated that topical inoculation of the rabbit cornea with the RE strain of HSV-1 induced 'non-specific' morphological damage to the endothelium as assessed by coelestine blue staining. Virus was cultured from endothelial cell scrapings prior to but not during the time of maximal corneal pathology. Owing to the nature of the culturing technique the source of virus could not be positively identified. In contrast to these studies other studies indicate that during periods of most severe inflammation virus antigens are rarely found in the endothelium following topical epithelial infection of the rabbit unless immunosuppressive agents are given.¹⁵

In the studies reported here intrastromal injection of the RE strain of HSV-1 resulted in the development of corneal oedema in rabbits peaking at a time after epithelial lesions were nearly healed and virus could not be detected in tears. The data show that stromal disease, as evaluated by the subjective scoring system devised by McNeill and Kaufman,¹⁶ correlates closely with corneal thickness as measured by pachymetry during the first 30 days after infec-

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tion. In the oedematous corneas no virus or viral antigen could be detected by transmission electron microscopy or immunocytochemistry. Corneal endothelial cell numbers and morphometric parameters such as hexagonality were not significantly altered during development and recovery from oedema, though many abnormalities in alizarin red staining were observed. At the time of maximal thickness what appeared to be inflammatory and immune cells were found integrated within the endothelial layer. The results show that corneal oedema can be induced after acute stromal and epithelial disease in the absence of viral cytolytic destruction of the endothelium.

Material and methods

HERPES SIMPLEX VIRUS CORNEAL INFECTIONS

Specific pathogen-free male New Zealand white rabbits (2.3–2.7 kg) (Hazleton Research Products, Denver, PA) free of pre-existing corneal defects were infected bilaterally by intrastromal injection of 20 µl of phosphate buffered saline (PBS) containing 10³ plaque forming units (pfu) of the RE strain of HSV-1.¹⁷ Control rabbits received intrastromal injections of 20 µl of the same dilution of mock antigen prepared from uninfected RK-13 cell lysate.

CLINICAL ASSESSMENT OF DISEASE

At selected daily intervals before and after infection, rabbits were anaesthetised by intramuscular injection of 5 mg of ketamine hydrochloride and 10 mg of xylazine. Corneal thickness was measured by use of an ultrasonic pachymeter (DGH Technology Inc, Frazer, PA) which was calibrated for use on rabbit corneas. Central corneal thickness was taken as the mean of three measurements for each cornea.

The severity of corneal stromal disease was graded on a 0 to 4 scale by means of a slit-lamp.¹⁶ Briefly, grading of disease severity was based on corneal clarity (0=clear cornea with iris details distinctly visible; 1=detectable oedema with iris details clearly visible; 2=gross oedema with iris details still distinctly visible; 3=gross oedema with iris details and pupillary border no longer distinctly visible; 4=opaque cornea with iris details not visible). Corneal epithelial disease was graded on a 0 to 4 scale following fluorescein staining (0=no stain; 1=25% of the corneal surface stained; 2=50% staining; 3=75%

Figure 1 Kinetics of development in stromal disease (Δ) and oedema (O) after intrastromal injection of HSV-1. Each data point represents the mean with the standard error of the mean. On days 0 through 8 (n=30 eyes), days 11 through 18 (n=14<22), and days 20 through 36 (n=10).

staining; 4=100% staining). Wide field specular micrographs were taken before and for up to three days after injection with a Keller-Konan specular microscope (Keeler Instruments, Inc, Broomall, PA) on Kodak Tri-X film which was developed and printed for morphometric analysis.¹⁸ Clusters of 100 individual cells were digitised and subjected to computer analysis to calculate cell density, cell area, coefficient of variation of cell area, and percentage of hexagonal cells.¹⁹

ALIZARIN RED S STAINING

At selected intervals following intrastromal injection rabbits were killed by intravenous injection of sodium pentobarbital and the corneas excised with a scleral rim. Flat mounts of whole corneas were prepared with the endothelial surface upwards so that the cell junctions would stain with alizarin red S as described by Taylor and Hunt.¹⁹ The stained endothelial flat mounts were photographed, prints prepared, and morphometric analysis performed.²⁰

PEROXIDASE/ANTIPEROXIDASE STAINING

A modification of the technique described by Shimeld *et al*²¹ was used to stain corneal endothelial flat mounts for the presence of HSV-specific antigens by the immunoperoxidase technique. Corneas were excised with 2–3 mm of scleral rim at various times after intrastromal injection and fixed with Bouin's solution for 12 h. Each cornea was rinsed in 50% ethanol and stored in 70% ethanol until staining. Prior to staining the corneal tissues were rehydrated, and a 10 mm central corneal button removed by trephination. The endothelium with a small amount of stroma was removed by stripping with forceps. The endothelial layers were mounted on slides and dried under coverslips at room temperature. The tissues were treated with 4–8% hydrogen peroxide in methanol, washed in phosphate buffered saline (PBS), and treated with normal goat serum (Calbiochem, La Jolla, CA). Tissues were then sequentially treated with appropriate dilutions of guinea-pig anti-HSV-1 antiserum (Bethesda Research Products, Bethesda, MD), affinity purified goat anti-guinea-pig IgG (Jackson Immuno-Research

Table 1 Morphometric parameters of the corneal endothelium following intrastromal injection mean (with SEM)

Days after injection	HSV*	Cells/mm ²	Mean cell area (µm ²)	Coeff. var†	% Hexagons	n‡
6	–	4128 (260)	244 (15)	16 (1)	71 (1)	3
	+	3652 (205)	279 (16)	18 (1)	71 (3)	6
12	–	3626 (348)	277 (8)	16 (1)	71 (3)	3
	+	3834 (147)	262 (10)	23 (2)	73 (3)	5
18	–	3989 (192)	252 (12)	22 (2)	66 (1)	3
	+	3828 (321)	265 (24)	18 (1)	67 (2)	3
27	–	3828 (131)	262 (9)	19 (1)	67 (2)	4
	+	3477 (146)	289 (12)	17 (1)	68 (3)	4

* – Indicates control corneas receiving injections of mock antigen preparation; + indicates HSV infected corneas. † Coefficient of variation of the mean cell area. ‡ Number of micrographs from different eyes which were digitised.

Figure 2 Alizarin red S stained flat mounts of the corneal endothelium of mock antigen-injected control eyes (top) and HSV-infected eyes (bottom) prepared 12 days after infection. Examples of staining abnormalities are indicated by arrows. (Original magnification $\times 132$.)

Laboratories, West Grove, PA), and guinea-pig peroxidase/antiperoxidase complex (Jackson Immuno-Research Laboratories, West Grove, PA). Coloured reaction product was detected by reaction with 0.05% diaminobenzoic acid in Tris-HCl buffer (pH 7.6) containing 0.03% hydrogen peroxide. Tissues were rinsed and a coverslip placed over them for observation.

MICROSCOPY

For scanning electron microscopy of the endothelium corneas were excised, fixed in 2.7% glutaraldehyde in phosphate buffer (pH 7.6),

Figure 3 Scanning electron micrographs of corneal endothelium of mock-antigen-injected control corneas (top), HSV-infected corneas six days after infection (centre), and 12 days after infection (bottom). Infiltrating cells on endothelial surface (*), junctional gaps (single arrows) and an area containing infiltrating cells within the endothelial cell layer (double arrows) were observed. (Original magnification $\times 500$.)

postfixed in 2% osmium tetroxide, mounted on stubs, and sputter coated with gold-palladium.²² Corneal tissues examined by scanning electron microscopy were removed from scanning electron microscopy mounts with acetone and immersed in propylene oxide to remove the gold-palladium coating. Each cornea was cut into segments and the segments were embedded in Spurr's low viscosity epoxy resin (Polysciences Inc, Warrington, PA). Sections (1 μ m) were cut, mounted on microscope slides, and stained with toluidine blue for light microscopy. Thin sections were cut from the same blocks, stained with uranyl acetate and lead citrate, and mounted on grids for transmission electron microscopy (TEM).²²

All numerical data are reported as the mean with standard error of the mean. Student's *t* test

or paired *t* test were used where appropriate to compare data of various groups.

Results

CLINICAL ASSESSMENT

The eyes of 15 rabbits were infected by intrasomal injection of 10^3 plaque forming units (pfu) of the RE strain of HSV-1. The progression of corneal disease was followed by slit-lamp examination and ultrasonic pachymetry. Corneal stromal disease as assessed by the subjective grading scheme of McNeill and Kaufman¹⁶ reached maximum about 12–15 days after infection (Fig 1). The measured corneal thickness similarly reached a maximum in that time frame (Fig 1). Subjective clinical evaluation and the actually measured corneal thickness showed a close relationship for 27 days after infection. Corneal epithelial disease in HSV infected rabbits as measured by fluorescein staining progressed steadily to a maximum score (1.1 (SEM 0.2) $n=30$) by eight days after infection and was nearly resolved by day 12 after infection (0.2 (SEM 0.1), $n=15$). Control rabbits receiving 20 μ l injections of equivalent dilution of mock antigen showed no significant increase in corneal thickness (384 (SEM 4) μ m, $n=16$) or stromal disease (0.1 (SEM 0.2) $n=16$) on day 13 after injection. These data predict that endothelial dysfunction or abnormality would be the greatest during the period 12–15 days after infection.

MORPHOMETRIC ANALYSIS OF THE CORNEAL ENDOTHELIUM

To insure that intrastromal injection of 20 μ l of either virus or mock antigen diluted in Hank's balanced salt solution did not cause direct cell damage, the endothelium was examined by contact specular microscopy. Eight eyes of randomly selected rabbits were photographed prior to injection and one day after injection. Prior to injection the endothelial layers contained 3513 (SEM 76) cells/ mm^2 with a mean area of 286 (SEM 6) μm^2 /cell having a coefficient of variation of 18 (SEM 2)%, and 71 (SEM 2)% of the cells were hexagons. One day after injection the central corneal endothelium in the area of the injection site contained 3414 (SEM 79) cells/ mm^2 with a mean area of 294 (SEM 7) μm^2 /cell having a coefficient of variation of 20 (SEM 3)% and 72 (SEM 2)% hexagons. A paired *t* test showed no

significant cell loss or other abnormalities, so that the injection procedure did not appear to result in endothelial cell damage.

Corneal thickness began to increase significantly by days 6–8 after infection, so clear, useful specular microscopic images of the endothelium were nearly impossible to obtain. We therefore assessed all endothelial morphometric parameters by preparation of alizarin red S stained flat mounts. The preparation and analysis of flat mounts of this nature are useful in obtaining morphometric analysis. However, owing to about 14% shrinkage, the apparent cell number is increased by about 14%.²⁰ Morphometric analysis of the corneal endothelium of mock-antigen-injected controls and HSV-1-infected corneas indicated that no significant loss of endothelial cells occurred either during the development or recovery from HSV-1 induced corneal oedema and stromal disease (Table 1). Similarly, because no significant change was observed in the coefficient of variation of cell size or the percentage of cells remaining as hexagons, there is no evidence of recent major endothelial cell loss in the vicinity of the areas digitised.¹⁸

The alizarin red-stained endothelial flat mounts taken during the period of maximal corneal thickness did show many staining abnormalities. The endothelium of corneas receiving injections of mock antigen displayed a normal staining pattern (Fig 2, top). The flat mounts of HSV-1 infected corneas, however, had numerous defects, including areas of pooling of stain at the cell vertices, areas of diffuse stain, areas of intense stain, and areas where the staining pattern of the endothelial cell borders was no longer visible (Fig 2, bottom). In the latter case it was difficult to determine the exact cause of the loss of the normal staining pattern. In many cases small areas appeared in which the normal staining pattern was faint but intact and obscured by debris or cells sitting on or within the endothelial layer.

SCANNING ELECTRON MICROSCOPY

In order to observe better the nature of the defects in the corneal endothelium, corneas of control and HSV-1 infected rabbits taken during each of four time periods were examined by scanning electron microscopy. Control corneas taken 6–8 days, 12–16 days, 18–20 days, and 30–37 days after injection of mock antigen showed few obvious defects ($n=3$ during each time

Figure 4 Toluidine blue-stained sections showing the endothelium prepared from HSV infected corneas taken 12 days after infection. Infiltrating cells were attached to the endothelial layer as indicated by the arrow (left) and incorporated within the layer as shown by the arrows (right). (Original magnification $\times 132$.)

Figure 5 Transmission electron micrograph shows the corneal endothelium from an HSV-1 infected rabbit 12 days after infection with an unidentified infiltrating cell (arrow) within the endothelial cell layer and adjacent to an endothelial cell (double arrows). (Original magnification $\times 5000$.)

period) (Fig 3, top). HSV infected corneas taken early in the course of development of stromal disease and oedema (6–8 days after infection) appeared to possess inflammatory cells in or on a normal endothelial cell layer ($n=6$) (Fig 3, centre). At severe stages of oedema numerous endothelial defects appeared, including junctional gaps and infiltration of the cell layer; however, major areas of endothelial cell loss were not observed ($n=5$) (Fig 3, bottom).

In random electron micrographs taken at magnification $\times 200$ and evaluated in a masked fashion the number of defects per field increased significantly in the HSV infected rabbits from 1.8 (SEM 0.9) defects/field ($n=4$) at days 6–7 after inoculation to a maximum of 27.6 (SEM 17.9) ($n=3$) at days 12–14, about the time of maximum corneal thickness. Numbers of defects then gradually decreased, with 6.8 (SEM 2.6) ($n=4$) at 18–20 days after infection and returned to 3.0 (SEM 2) ($n=3$) after 30–37 days. Corneas from control animals taken at each of these time points showed no comparable defects.

LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

In an effort to understand better the nature of the

endothelial defects in the corneas observed by SEM the specimens were uncoated, reblocked in epoxy resin, and sectioned for both light and TEM. Observation of these specimens by light microscopy following toluidine blue staining suggested that the irregularities in the endothelium during the period of maximum corneal oedema were due to infiltration of the endothelial layer with inflammatory cells and lymphocytes (Fig 4). Early in the course of oedema (6–8 days after infection), cells were frequently observed attached to the luminal surface of the endothelium. As oedema became more severe, TEM indicated that infiltrating cells were incorporated within the endothelial layer, especially during the period of maximal corneal oedema (Figs 5, 6, 7). Many of the cells had large areas of rough endoplasmic reticulum typical of plasma cells (Fig 7). Cells were not observed within or spanning Descemet's membrane. Cells and flare in the anterior chamber were rarely observed at any stage of disease. An intense anterior chamber reaction characterised by the development of hypopyon was not characteristic of the disease studied.

Virus particles were not detected by TEM in any endothelial cell layers observed (4–6 corneas

Figure 6 Transmission electron micrograph shows an unidentified infiltrating cell (arrow) incorporated within the endothelial cell layer adjacent to an endothelial cell (double arrows) in the cornea of an HSV-1 infected rabbit 14 days after infection. (Original magnification $\times 8000$.)

Figure 7 Transmission electron micrograph of a plasma cell (asterisk) adjacent to an endothelial cell in the cornea of an HSV-1 infected rabbit 12 days after injection. (Original magnification $\times 5000$.)

examined per time point, four blocks per cornea, 6–10 sections per block). Sections of corneas taken at each time period from six days after infection to 36 days after infection showed no evidence of replicating virus in the endothelium. Virus particles were found with ease in the epithelium and stroma from corneas taken six days after infection. The presence of non-enveloped particles within the nuclei and enveloped particles in the cytoplasm suggested that virus was replicating in both stromal and epithelial cells. By day 12 after infection virus particles became increasingly difficult to locate in these cells until by days 30–36 after infection virus was not observed in any corneas.

IMMUNOPEROXIDASE DETECTION OF HSV-1 ANTIGEN

Endothelial flat mounts of Bouin's fixed corneas were prepared from each of the groups of rabbits studied to detect the presence of viral antigen. Monolayers of Vero cells and organ cultured corneas were infected *in vitro* to serve as antigen-positive control. Peroxidase reaction product was readily visible in positive controls and was not visible in virus negative controls. None of the eight endothelial layers examined from infected rabbits taken 6–8 days after infection or the nine corneas taken during the period of maximum oedema possessed peroxidase-positive endothelial cells. Corneas taken after day 12 after infection often contained peroxidase-positive cells, thought to be plasma cells (Fig 8). These cells appeared with greatest frequency during the 12–18 day period after infection and decreased but were still occasionally present in some endothelial preparations as late as 36 days after infection. Immunoperoxidase-positive cells were not observed in mock-antigen-injected control cultures.

Discussion

Corneal oedema is associated with a diversity of clinical presentations of ocular HSV infections. Dysfunction of the corneal endothelium is the presumed source of oedema, but more than one mechanism may lead to this dysfunction. We have shown that the RE strain of HSV-1 used in

these studies does not readily disseminate within the cornea to infect the endothelium. Alizarin red S staining and scanning electron microscopy indicate that, although defects could be detected, the corneal endothelial cell layer remains basically intact. Nevertheless corneal oedema is a significant feature of the disease, and its appearance closely correlates with the clinical evaluation of severity of stromal disease and infiltration of inflammatory and immune cells into the endothelium. Under these conditions of inflammation and oedema neither virus nor viral antigens were found in association with the corneal endothelium. While it is possible that the levels of virus and viral antigen are below the level which we can detect, the presence of an endothelium devoid of large areas of cell destruction makes it unlikely that the endothelial cells are a principal site of virus replication. Therefore with the strain of HSV used in these studies even after intrastromal injection oedema occurs, but the virus does not appear to move to the endothelium.

Infection of rabbits with other strains of virus which can disseminate in the cornea to the endothelium appears to produce oedema by a second mechanism. Infection of endothelial cells followed by cell lysis could clearly compromise endothelial barrier function. In animal experiments virus has been observed to infect endothelial cells after direct injection into the anterior chamber of rabbits,^{10,11} and selected isolates of some strains of virus such as McKrae and RE appear to be capable of spreading to the endothelium.^{11,14} In the latter case the data provided do not definitely identify the corneal endothelial cells as supporting virus replication.

Data from patients are limited by the inability to obtain samples or visualise the endothelium during severe oedema. While virus isolation from the anterior chamber has been reported, the source of virus has not been positively identified as endothelial cells.⁷

Direct observation of the endothelium of some patients with HSV induced disease has provided suggestive evidence of focal endothelial cell loss, but the cause of the apparent cell loss cannot be directly established.^{4,8,9} Other endothelial cell abnormalities in the form of multinucleate giant cells and increased mean cell area have been observed in one patient with a 12-year history of

Figure 8 Immunoperoxidase stained and hematoxylin counterstained flat mount of the corneal endothelium of an HSV-1 infected rabbit taken 19 days after infection. The endothelial layer contained both immunoperoxidase-positive plasma cells (arrow) and immunoperoxidase-negative infiltrating cells (nuclei stained with haematoxylin, double arrows) and endothelial cells (asterisks.) (Original magnification $\times 400$.)

chronic HSV disease, while a few significant morphometric changes were observed in the endothelium of another patient.²³

Our data provide evidence to support a mechanism by which HSV induces corneal oedema without virus destruction of endothelial cells. The data show the development and recovery from corneal oedema without measurable loss of endothelial cells or major changes in morphometric parameters. The isolate of HSV-1 (RE) used in these studies as well as the HF and Walker strains of HSV-1 have been shown to be unable to disseminate within the cornea. These strains of virus, however, induce corneal oedema.^{11 15 24} The inflammatory disease induced by HSV-1 may result not from loss of corneal endothelial cells but rather from loss of cell function. It is not clear whether barrier or 'pump' function or both may be altered owing to HSV infection. Decompensation of corneal endothelial function due to infiltration with inflammatory and immune cells may be responsible for the oedema. The presence of these cells suggests that chemotactic factors as well as inflammatory mediators and cytokines are present in the cornea. Metabolites of arachidonic acid which contribute to inflammation have been shown to be produced within the cornea during HSV-1 infection.²⁵ Further studies will be required to determine the identity of cytokines and mediators of inflammation present in HSV infected corneas in order to determine their role in corneal oedema.

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