Published in final edited form as:

Ophthalmology. 2009 July ; 116(7): 1301–1305. doi:10.1016/j.opthta.2009.03.031.

HSV KERATITIS: HISTOPATHOLOGIC INFLAMMATION AND CORNEAL ALLOGRAFT REJECTION

Roni M. Shtein, MD^{1,*}, Denise D. Garcia, MD¹, David C. Musch, PhD^{1,2}, and Victor M. Elner, MD, PhD^{1,3}

¹ Department of Ophthalmology and Visual Sciences

² Department of Epidemiology

³ Department of Pathology University of Michigan, Ann Arbor, Michigan

Abstract

Objective—To identify whether histopathologic and immunoassay biomarkers of inflammation are predictive for allograft rejection following penetrating keratoplasty for herpes simplex virus (HSV) keratitis.

Design—Retrospective, interventional case series with prospective component of pathologic evaluation of frozen tissue.

Participants—Sixty-two consecutive patients with HSV keratitis who underwent penetrating keratoplasty.

Methods—A chart review and histopathologic examination of the excised host corneal button was performed to identify associations between clinical data and histopathologic presence of inflammation. Enzyme-linked immunosorbent assay (ELISA) for interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) chemokines and immunohistochemical staining for HLA-DR and intercellular adhesion molecule-1 (ICAM-1) antigens was also performed in inflamed and non-inflamed specimens.

Main Outcome Measure—To determine whether the presence of subclinical inflammation at the time of penetrating keratoplasty predicts allograft rejection.

Results—While 81% of patients had clinically quiescent disease, histopathology revealed that 74% had active corneal inflammation, a finding that was associated with the presence of clinical neovascularization ($P = .01$). Allograft rejections were experienced by 34% of the patients in this cohort. The histopathologic presence of inflammation was a risk factor for allograft rejection ($P = .02$). Corneal specimens demonstrating inflammation had significantly increased IL-8 ($P = 0.0005$) and MCP-1 ($P = 0.003$), and greater immunoreactivity for HLA-DR and ICAM-1 when compared to specimens without inflammation. IL-10 treatment *ex vivo* significantly inhibited IL-8 ($P = .006$),

Address correspondence to: Victor M. Elner, MD, PhD, University of Michigan, Kellogg Eye Center, 1000 Wall Street, Ann Arbor, Michigan 48105, (734) 764-4182, FAX (734) 936-3815; velner@umich.edu.

This manuscript is based on a presentation at the Annual Meeting of the American Ophthalmological Society and subsequently published in the Transactions of the American Ophthalmological Society in 2008. The manuscript underwent subsequent peer review by the Ophthalmology and has been modified following the peer review process.

Conflict of Interest: No author has a financial/conflicting interest

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

and MCP-1 ($P = .01$) chemokines, and qualitatively substantially reduced HLA-DR, but not ICAM-1 expression.

Conclusion—Histopathologic inflammation is a risk factor for corneal allograft rejection.

Patients undergoing penetrating keratoplasty (PKP) for sequelae of herpes simplex virus (HSV) keratitis are at higher risk for adverse corneal allograft outcomes when compared to individuals undergoing grafting for conditions such as keratoconus and Fuchs' corneal dystrophy.^{1,2} The post-operative course can be complicated by high rates of HSV recurrence, graft rejection, and graft failure.^{3–6} To identify whether histopathologic inflammation predicts graft rejection, we examined corneal tissue from patients with HSV keratitis who underwent PKP for visual rehabilitation.

We hypothesize that patients may have subclinical corneal inflammation in spite of the clinical appearance of quiescent HSV disease and that this inflammation in the hosts' corneal tissue places allografts in these surgical beds at risk for rejection. By examining host corneal tissue removed at the time of surgery, we determined whether inflammation is an important histopathologic feature that identifies patients at high risk for graft rejection. To improve understanding of the possible pathophysiologic mechanisms of HSV keratitis, we also examined corneas for functional biomarkers of inflammation. We measured the two major leukocyte chemoattractants interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), and the expression of HLA-DR and intercellular adhesion molecule-1 (ICAM-1), two key receptors on cells mediating immune mechanisms, in specimens with and without histopathologic inflammation. In these specimens, the effect of IL-10, a multifunctional cytokine shown to suppress HSV keratitis in animal models,⁷ was incubated with portions of the specimens to determine its ability to suppress chemokine and receptor expression.

METHODS

PATIENTS

All PKP's performed for sequelae of HSV keratitis at the University of Michigan from August 1990 to December 2000 were assessed for inclusion in the study. Inclusion criteria included primary PKP performed in corneas without clinically active disease for any sequelae of HSV keratitis (epithelial, stromal, keratouveitic, or any combination). A total of 79 allografts were performed on 73 patients in this time period. Data were not available for 3 patients. Six patients were grafted twice during this time period and only their first grafts were eligible for inclusion. Eight other patients had primary grafts done prior to 1990, and had subsequent grafts done during our study period. These repeat keratoplasties were excluded, leaving 62 primary grafts in this study. All surgeries were performed by corneal subspecialists. Charts were reviewed for the following information: disease-free time before surgery, allograft rejection episodes, HSV recurrence, and histopathologic presence of inflammation and inflammatory biomarkers in the excised corneal tissue. This study received Institutional Review Board approval at the University of Michigan Medical Center.

Graft rejection was defined by an anterior chamber reaction with keratic precipitates (KP) on the donor endothelium only, by an endothelial or epithelial rejection line, or by graft edema with associated KP on the donor endothelium. Active HSV keratitis was defined by the presence of dendritic or geographic epithelial keratitis, and/or ulceration. HSV keratouveitis was defined by the presence of KP on both the donor and host endothelium. Clinical quiescence of HSV infection was defined as no change on clinical examination for at least 6 months.

Postoperative oral acyclovir prophylaxis was prescribed in 51 (85%) of the 62 patients. The initial dose used was variable, as was the tapering regimen, however, patients were on at least 800mg per day for an average of 6 months, and at least 400mg per day for an average of 17

months. Postoperative topical prednisolone acetate 0.1% eye drops (averaging 4 times daily and subsequently tapered) were used in all patients. Episodes of HSV recurrence were treated with oral acyclovir or trifluridine eye drops (Viroptic, Glaxo Wellcome). Episodes of rejection were treated with prednisolone acetate 1% eye drops, tapered over several weeks.

PATHOLOGY

Each specimen removed from all 62 patients was examined grossly for regions of maximal vascularization, opacity, and variations in thickness. The specimen was then bisected along a secant 0.5 mm from and parallel to the diameter demonstrating, in order, maximal vascularization, opacity, or variable thickness. After routine processing, six micron paraffin step sections were obtained at 100 micron intervals for 1mm of the specimen, straddling the diameter of maximal gross pathology. The paraffin sections were stained with hematoxylin and eosin. The sections from each specimen were evaluated and graded in the week subsequent to its removal by an ophthalmic pathologist (VME) who was masked as to all clinical details except for the diagnosis. Each of the 62 specimens was rendered a pathologic diagnosis and graded for the presence or absence of inflammation. The presence of inflammation was confirmed by identifying any scattered or focal collections of leukocytes, or more extensive leukocyte infiltration.

Prior to the above described routine processing, a segment of fresh tissue from twenty-four of the specimens was removed and bisected. Portions from each specimen were submerged in media alone or in media containing IL-10 (100 ng/mL) at 37 C for 24 hours. The portions of untreated and IL-10 treated cornea were then frozen in separate containers at -70 C. Of these frozen tissue specimens, eight with and eight without histopathologic inflammation were thawed and processed for IL-8 and MCP-1 enzyme-linked immunosorbent assay (ELISA) as previously described.⁸ The remaining four specimens with inflammation and four without inflammation were frozen in OCT compound for immunohistochemical staining, as previously described.⁹ Sections cut from these frozen specimens were placed on glass slides coated with poly-L-lysine and were processed for immunohistochemical staining for HLA-DR and ICAM-1, as previously described.⁹ The immunohistochemically stained tissue sections were graded as 0 (no visible staining), 1+ (intense staining in < 25% of cells), 2+ (intense staining in < 50% of cells), 3+ (intense staining in < 75% of cells), or 4+ (intense staining in > 75% of cells), as previously described.⁹

STATISTICAL ANALYSIS

The clinical and histopathologic data were analyzed using the chi square test, Fisher's exact test, analysis of variance, Kaplan-Meier survival curves, the log rank test, and Cox regression. ELISA results were analyzed by t-test with equal (IL-8) or unequal (MCP-1) variance and paired t-test (IL-10 inhibition). Unless otherwise indicated, data are given as mean + SD. SAS 9.0 statistical software (SAS Institute, Cary, NC) was used for the data analyses and comparisons.

RESULTS

The average patient age at surgery was 55 + 22 years (range, 5–85 years) and the average disease duration was 19 + 12 years (range, 0.25 to 72 years). Fifty-three percent of patients were female. The average duration of clinical quiescence before surgery was 50 + 78 months (range, 3–360 months). Average follow-up was 43 + 32 months (range, 3–142 months). Twenty-one (34%) of the patients in this cohort experienced an allograft rejection episode. The average time from surgery to allograft rejection episode was 12 + 16 months (range, 1–69 months). Indications for surgery were corneal scarring in 60 (97%) patients, descemetocoele in 1 (1.5%), and perforation in 1 (1.5%). Nine patients had an HSV recurrence in their allograft

during the study follow-up; 1 patient manifested with keratouveitis, 1 with geographic epithelial keratitis, and the remaining 7 with dendritic epithelial keratitis. Six (67%) of the 9 patients with HSV recurrence had also experienced an allograft rejection episode.

In spite of the fact that 50 (81%) of the patients had clinically quiet HSV disease for more than 6 months prior to PKP, only 16 (26%) patients had no histopathologically visible inflammation; the rest had some degree of inflammation present. The histopathologic presence of inflammation was associated with the presence of clinical neovascularization preoperatively ($p = .01$). Of the 16 patients without any histopathologic inflammation in their corneas, only 1 (6.3%) experienced an allograft rejection. On the other hand, 20 (43.5%) of the 46 patients with histopathologic inflammation experienced a rejection. Figure 1 shows a Kaplan-Meier time to allograft rejection analysis in these two groups ($p = .02$, log-rank). The duration of clinical quiescence prior to PKP did not correlate with development of allograft rejection ($p = 0.84$).

The eight corneal specimens with histopathologic inflammation had IL-8 (38 ± 15 ng/mg tissue) and MCP-1 (4.9 ± 2.3 ng/mg tissue) that was significantly greater than IL-8 (7.9 ± 10 ng/mg tissue) and MCP-1 ($1.4 \pm .85$ ng/mg tissue) in specimens with no visible inflammation (IL-8: $p = 0.0005$; MCP-1: $p = 0.003$) (Figure 2). The four inflamed specimens treated with media containing IL-10 (100ng/mL) demonstrated significant inhibition of IL-8 ($82 \pm 14\%$, $p = 0.006$) and MCP-1 ($54 \pm 16\%$, $p = 0.01$) compared to tissue treated with media alone (Figure 3).

HLA-DR and ICAM-1 immunoreactivity, ranging from 2–3+ positivity in all four specimens with inflammation, was substantially greater than the 0–1+ positivity in specimens lacking visible inflammation. Exogenous IL-10 substantially reduced HLA-DR staining in the inflamed tissues to 1–2+ staining (Figure 4). However, IL-10 had no effect on the amount of ICAM-1 immunopositivity (not shown).

DISCUSSION

To our knowledge, there are no other published studies examining the relationship between histopathology of excised host corneal tissue and subsequent allograft outcomes. One study published in 2004 by Branco and associates,¹⁰ looked at the records of all corneal tissue submitted from 1972 to 2001 to the pathology laboratory at the University of California at San Francisco. There were 4,207 grafts performed, 76 (1.8%) of which were for HSV keratitis. They reported on the pathological findings in corneas with a clinical diagnosis of HSV keratitis including inflammatory cells in 87%. The authors did not comment on what effect the presence of the histopathologic findings had on subsequent allograft outcomes.

The significance of the histopathologic, immunohistochemical and ELISA results in this study is emphasized by the fact that there were no statistically significant clinical variables predictive for allograft rejection in this cohort.¹¹ Our analysis of the tissue removed from these patients at the time of PKP reveals that sub-clinical inflammation predicts rejection. These findings are of practical significance to the clinician in care of patients after PKP.

Despite the fact that 81% of patients demonstrated clinically quiescent disease for at least 6 months, 74% had inflammation on histopathologic evaluation of their corneal tissue. This supports our hypothesis that inflammation exists even when clinical signs are absent. Inflammation within the clinically quiescent corneal tissue probably reflects the putative mechanisms of host immune responses to residual viral antigens or virally-altered cell proteins as propagators of inflammation even after successful clearance of intact virus.^{12,13} Our immunohistochemical and chemokine data of the inflamed corneal tissues improves our understanding of the corneal inflammatory response to HSV infection. We previously showed

HLA-DR and ICAM-1 expression to be increased in HSV stromal keratitis¹⁴ and demonstrated reduced expression of HLA-DR, but not ICAM-1 due to IL-10 treatment,⁹ findings that were confirmed in this study. We now also show that there are substantial levels of leukocytic chemokines (IL-8 and MCP-1) in corneas with clinically quiescent HSV stromal keratitis. These chemokines are known to attract and stimulate various leukocyte subsets and their presence is likely to participate in the perpetuation of the stromal disease.^{15,16}

Studies in murine models have shown that IL-10 has the ability to lessen the severity of HSV keratitis without impairing viral clearance or reducing host resistance to the virus.⁷ Initial observations in a murine model of HSV keratitis suggested that MCP-1, a mononuclear phagocyte chemokine, did not play an important role.¹⁷ This may be due to the fact that a neutrophil response predominates in the murine model and appears to be driven by neutrophil chemokines, principally MIP-2.¹⁷ A subsequent paper, however, showed that even in the murine model, in which mononuclear phagocytes comprise only a minority of the corneal cell infiltrate, there is some protective effect of MCP-1 against the development of HSV keratitis.¹⁶

In humans, HSV stromal keratitis is characterized by a mixed infiltrate composed of chronic inflammatory cells, including lymphocytes, neutrophils, and mononuclear phagocytes.¹⁸ **As expected, the inflamed tissues examined in this study all exhibited inflammatory infiltrates composed principally of chronic inflammatory cells, with lesser numbers of neutrophils.** Corresponding to the histopathologic findings in human disease, we found that both IL-8, a neutrophil and lymphocyte chemokine, and MCP-1, a mononuclear phagocyte chemokine, were elevated in our samples. In addition, both were substantially suppressed by *ex vivo* IL-10 treatment of the excised corneal buttons that demonstrated inflammation, histopathologically. IL-8 and MCP-1 are two principle cytokines that elicit inflammatory cells to enter corneal tissue during inflammation.^{15,16} Further, our observations of IL-10 effects on IL-8, MCP-1, and HLA-DR raise the possibility that IL-10 is a potential therapeutic agent to reduce the severity of keratitis in humans while permitting viral clearing as it does in the murine model.

Corneal allografts are unlike other solid organ transplants in that allograft tissue is placed in a bed of the host's residual diseased tissue. This may predispose the allograft to adverse outcomes, such as rejection. Pathogenetically, the presence of inflammation, which we found to correlate with subsequent graft rejection, may be due to the fact that such inflammation in HSV keratitis is associated with increased corneal expression of HLA-DR antigens and ICAM-1.^{9,14,19} In this study, expression of these markers was found preferentially at sites of active keratitis and correlated with the presence of inflammation. Expression of these molecules is known to enhance antigen recognition and subsequent allograft rejection which we found to correlate with the presence of inflammation.¹⁴ Although these grafts were prone to rejection, careful follow up and intensive therapy of rejection episodes was able to preserve functioning grafts in many cases.^{11,20} **It is also possible that intensive preoperative anti-inflammatory treatment would reduce the risk of subsequent allograft rejection.**

Despite the retrospective nature of this study, the histopathologic presence of leukocyte infiltration, and the immunohistochemical findings regarding HLA-DR, ICAM-1, IL-8, and MCP-1 in the removed host corneal tissue were well defined, as was the clinical endpoint of allograft rejection. This lends confidence that the conclusions drawn from the study are likely to be true and clinically relevant. Moreover, as the histopathologic findings were determined in tissue grossed and processed in the usual fashion for corneal surgical pathology specimens, the observations made are relevant to routine clinical practice. This establishes a role for the pathologist in assisting the clinician in their choice of postoperative patient management.

Acknowledgments

Financial Support: This study was supported by EY017885 (RMS) and EY7003 and EY9441 (VME); Dr. Elner is the recipient of a Senior Scientific Award from Research to Prevent Blindness.

References

1. Epstein RJ, Seedor JA, Dreizen NG, et al. Penetrating keratoplasty for herpes simplex keratitis and keratoconus: allograft rejection and survival. *Ophthalmology* 1987;94:935–44. [PubMed: 3309775]
2. Thompson RW Jr, Price MO, Bowers PJ, Price FW Jr. Long-term graft survival after penetrating keratoplasty. *Ophthalmology* 2003;110:1396–402. [PubMed: 12867398]
3. Cohen EJ, Laibson PR, Arentsen JJ. Corneal transplantation for herpes simplex keratitis. *Am J Ophthalmol* 1983;95:645–50. [PubMed: 6342400]
4. Foster CS, Duncan J. Penetrating keratoplasty for herpes simplex keratitis. *Am J Ophthalmol* 1981;92:336–43. [PubMed: 7027796]
5. Lomholt JA, Baggesen K, Ehlers N. Recurrence and rejection rates following corneal transplantation for herpes simplex keratitis. *Acta Ophthalmol Scand* 1995;73:29–32. [PubMed: 7627755]
6. Ficker LA, Kirkness CM, Rice NS, Steele AD. Long term prognosis for corneal grafting in herpes simplex keratitis. *Eye* 1988;2:400–8. [PubMed: 3075567]
7. Tumpey TM, Elner VM, Chen SH, et al. Interleukin-10 treatment can suppress stromal keratitis induced by herpes simplex virus type 1. *J Immunol* 1994;153:2258–65. [PubMed: 8051423]
8. Elner VM, Burnstine MA, Strieter RM, et al. Cell-associated human retinal pigment epithelium interleukin-8 and monocyte chemotactic protein-1: immunochemical and in-situ hybridization analyses. *Exp Eye Res* 1997;65:781–9. [PubMed: 9441701]
9. Boorstein SM, Elner SG, Meyer RF, et al. Interleukin-10 inhibition of HLA-DR expression in human herpes stromal keratitis. *Ophthalmology* 1994;101:1529–35. [PubMed: 7916449]
10. Branco BC, Gaudio PA, Margolis TP. Epidemiology and molecular analysis of herpes simplex keratitis requiring primary penetrating keratoplasty. *Br J Ophthalmol* 2004;88:1285–8. [PubMed: 15377552]
11. Garcia DD, Farjo Q, Musch DC, Sugar A. Effect of prophylactic oral acyclovir after penetrating keratoplasty for herpes simplex keratitis. *Cornea* 2007;26:930–4. [PubMed: 17721290]
12. Heiligenhaus A, Bauer D, Zheng M, et al. CD4+ T-cell type 1 and type 2 cytokines in the HSV-1 infected cornea. *Graefes Arch Clin Exp Ophthalmol* 1999;237:399–406. [PubMed: 10333107]
13. Pepose JS. Herpes simplex keratitis: role of viral infection versus immune response. *Surv Ophthalmol* 1991;35:345–52.
14. Elner VM, Dutt S, Pavilack MA, et al. Intercellular adhesion molecule-1 (ICAM-1) and HLA-DR antigens in herpes keratitis. *Ophthalmology* 1992;99:1400–7. [PubMed: 1357614]
15. Elner VM, Strieter RM, Pavilack MA, et al. Human corneal interleukin-8: IL-1 and TNF-induced gene expression and secretion. *Am J Pathol* 1991;139:977–88. [PubMed: 1951636]
16. Kim B, Sarangi PP, Lee Y, et al. Depletion of MCP-1 increases development of herpetic stromal keratitis by innate immune modulation. *J Leukoc Biol* 2006;80:1405–15. [PubMed: 16997857]
17. Tumpey TM, Cheng H, Yan XT, et al. Chemokine synthesis in the HSV-1 infected cornea and its suppression by interleukin-10. *J Leukoc Biol* 1998;63:486–92. [PubMed: 9544579]
18. Liesegang TJ. Ocular herpes simplex infection: pathogenesis and current therapy. *Mayo Clin Proc* 1988;63:1092–105. [PubMed: 2848161]
19. Dennis RF, Siemasko KF, Tang Q, et al. Involvement of LFA-1 and ICAM-1 in the herpetic disease resulting from HSV-1 corneal infection. *Curr Eye Res* 1995;14:55–62. [PubMed: 7720405]
20. Sangwan VS, Ramamurthy B, Shah U, et al. Outcome of corneal transplant rejection: a 10-year study. *Clin Experiment Ophthalmol* 2005;33:623–7. [PubMed: 16402956]

Figure 1. Rejection of corneal allografts in patients with **herpes simplex virus** (HSV) stromal keratitis. Kaplan-Meier survival curves of allograft rejection in patients with and without inflammation on histopathologic evaluation of their excised corneal tissue.

Figure 2. Interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) in corneal tissue with herpes simplex virus (HSV) stromal keratitis IL-8 (left) and MCP-1 (right) enzyme-linked immunosorbent assay (ELISA) of corneal tissue with (+) and without (-) visible inflammation.

Figure 3. **Interleukin-8** (IL-8) and **monocyte chemotactic protein-1** (MCP-1) in corneal tissue with **herpes simplex virus** (HSV) stromal keratitis treated with **interleukin-10** (IL-10). Percent inhibition of IL-8 (left) and MCP-1 (right), as measured by **enzyme-linked immunosorbent assay** (ELISA), of corneal tissue with inflammation after treatment with IL-10 *ex vivo*.

Figure 4. HLA-DR immunoreactivity in **herpes simplex virus** (HSV) stromal keratitis treated with **interleukin-10** (IL-10). Immunoreactivity for HLA-DR antigens in corneal stroma with inflammation is less in *ex vivo* IL-10-treated (1+, bottom) than untreated (2+, top) tissue. (Hematoxylin counterstain x160)