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HSV-1 antigens and DNA in the corneal explant buttons of patients with non-herpetic or clinically atypical herpetic stromal keratitis

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Abstract *Background:* Little is known about the role of HSV-1 in keratitis not primarily attributed to herpetic origin. This study therefore aimed to prospectively evaluate the corneal explant buttons of patients with non-herpetic or clinically atypical herpetic stromal keratitis (experimental group: non-HSK) for the presence of HSV-1 antigens and DNA, and to compare the findings with those from individuals with typical herpetic stromal keratitis (positive control group: HSK) or non-inflammatory degenerative keratopathy (negative control group). *Methods:* Corneal buttons derived from 51 patients with HSK, from 72 with non-HSK and from 30 with degenerative keratopathy were prospectively collected and subjected to immunohistochemical analysis for HSV-1 antigens and to HSV-1 DNA amplification. *Results:* In corneal buttons derived from patients with non-HSK, viral antigens were detected immunohistochemically in 8/72 cases and DNA amplified in 16/72. Corresponding values for the HSK group were 16/51 and 11/51. Taking viral antigen and DNA findings together, HSV-1 was detected in 18/72 (25%) patients with non-HSK and in 19/51 (37%) with HSK (p=0.2), but in only 2/30 (6%) individuals with non-inflammatory degenerative keratopathy. *Conclusion:* Since the detection frequencies for HSV-1 antigens and DNA were comparable in the HSK and non-HSK groups, Herpes may play an underestimated and as yet undefined role in non-herpetic and clinically atypical herpetic stromal keratitis, either as a primary trigger of the disease or as a secondary contributor to it. In this category of individuals, early antiherpetic therapy should be considered if patients do not respond in the expected manner to treatment for non-herpetic stromal keratitis.

Introduction

According to accepted concepts, recurrences of herpetic stromal disease are triggered by the reactivation of latent virus within the trigeminal ganglion or its periphery, this event being followed by transneuronal spread to the cornea [11, 26]. Nevertheless, viral DNA has been detected within the cornea even when there exists no evidence of active or previous clinical disease [32]. The possibility of corneal viral latency [9, 28, 33] and of persisting replicativity at various peripheral sites [34, 43], including the cornea [13], has added to the complexity of the picture without shedding further light on the disease mechanisms.

In cases of active stromal keratitis, replication has been localized to the corneal stroma by the direct electron-microscopic demonstration of complete viral particles therein [11]. It has been speculated that the presence of viral particles within the cornea triggers a specific local immune response which, in the initial phase, is directed against the virus itself [16, 38, 44]. At a later stage of the disease, and in chronic or recurrent inflam-

mation, keratitis is more likely to result from an immunopathological process triggered by neutrophils and orchestrated by CD4-positive T-lymphocytes [25]. It remains a matter of debate whether a failing autoimmunoregulation underlies this process or whether the inflammatory activity is redirected in targeting as yet undefined cross-reactive but common antigenic epitopes of HSV-1 within stromal structures [3], thereby inducing a stromal autoreactive process [40, 45]. By this means, the inflammatory process would become independent of viral replication.

In humans, primary ocular HSV-1 infection is not unusually subclinical [31], whereas the course followed by chronic and recurrent keratitis depends heavily upon the extent of stromal involvement, neovascularization and cicatrization [20].

Both amplification of HSV-1 DNA by polymerase chain reaction (PCR) and the immunohistochemical localization of viral antigens have proved to be sensitive means of detecting HSV-1 activity [21]. Accordingly, the aetiologic diagnosis of viral diseases such as necrotizing herpetic retinopathies may be established in up to 90% of cases by analysing samples of aqueous humour [14]. In instances of recurrent stromal keratitis, however, neither antigen detection nor the amplification of viral DNA by PCR has proved to be sufficiently sensitive [16, 19, 37]. The fact that even highly sensitive techniques have consistently failed to confirm the aetiology in cases of herpetic stromal keratitis may be accounted for by one of three hypotheses. It is possible, of course, that the clinical diagnosis is false. Alternatively, the replication of HSV-1 could lie below the level of detection. On the other hand, the low copy numbers of viral antigen and DNA could have been destroyed by local protease and DNAse activity. In this case, HSV-1 would play a subordinate role at the time of clinical diagnosis, but would nevertheless be capable of initiating a T-cell-directed immunopathological response even in the later absence of viral replication. This latter theory is currently gaining credence amongst virologists and immunologists in the field [33, 40].

We still have no clear conception of the manner in which HSV-1 drives herpetic keratitis. Viral replication probably takes place predominantly within the corneal epithelium, whilst the inflammatory reaction is expressed almost exclusively within the underlying stroma [40]. The enigma lies in the circumstance that viral replication is a prerequisite for the induction of inflammation [2].

HSV-1 has been reported to be present within both inflamed and clinically healthy corneal tissue [32], thereby raising doubt as to the clinical relevance of its detection. In the present study, we attempted to define the role of HSV-1 in cases that were not clinically attributable to this virus or not unambiguously so. This category of individuals was referred to as the non-herpetic stromal keratitis (non-HSK) group. Findings relating to the detection rates of HSV-1 antigens and DNA in this non-HSK group were compared to those in individuals with either clinically typical herpetic stromal keratitis (HSK group; positive control) or non-inflammatory degenerative keratopathy (negative control).

Materials and methods

A total of 153 corneal buttons were obtained from patients undergoing keratoplasty. Of these, 51 had clinically typical HSK [group 1 (positive control): HSK] and 72 had stromal keratitis which was not clinically attributable to HSV-1 or not unambiguously so [group 2 (experimental): non-HSK]; the other 30 had non-inflammatory degenerative keratopathy [group 3 (negative control)]. Corneal buttons were included in groups 1 or 2 irrespective of the level of disease activity. Our justification for this is that viral antigens and/or DNA presumably persist in the cornea for a lifetime, thus rendering their detection independent of disease activity. This concept of long-term persistence and latency of viral antigens and DNA within corneal tissue has only recently been reinforced [5, 7, 9, 12, 35]. Until now, there has existed no evidence for an association between the detection rates of viral DNA and/or antigens and clinical disease activity. Nevertheless, the vast majority of patients in groups 1 and 2 did not manifest marked inflammatory activity at the time of corneal transplantation.

Experimental and control groups

Group 1 (positive control), HSK. The diagnosis of HSK was established if a systemic granulomatous disease condition had been ruled out anamnestically in individuals with four or more of the eight criteria listed in Table 1. The anamnestic exclusion of systemic granulomatous disease was targeted to rule out ocular involvement in untreated or insufficiently treated cases of mycobacterial, sarcoidotic or syphilitic disease, which might lead independently to an unsatisfactory postoperative course.

Group 2 (experimental), non-HSK. The diagnosis of a non-herpetic disease condition was established in cases of stromal keratitis that did not meet the inclusion criteria for group 1, i.e. in patients whose clinical picture was not typically herpetic, and in those whose condition had been proved to be of other origin (Table 2).

Table 1 Diagnostic criteria for HSK

History of recurrent stromal keratitis
History, or documented episodes, of dendritic epithelial keratitis
Recurrent disciform keratitis with endothelial precipitates
Recurrent interstitial keratitis with deep stromal vascularization or acute stromal necrosis
Corneal hypaesthesia
Post-inflammatory sectorial atrophy of the iris after sectorial granulomatous iritis
Secondary glaucoma
Documented response to combined systemic Acyclovir and local steroid treatment in the past

Table 2 Diagnostic criteria for non-HSK

Inflammatory corneal disease that fails to meet the criteria for herpetic keratitis, i.e.
Scar after interstitial or parenchymatous keratitis without recurrences
Keratitis in systemic rheumatic and autoimmune disease
Viral keratitis of proven non-herpetic (i.e. varicella-zoster) origin
Proven bacterial or mycotic keratitis without recurrence
Excluded: Scarring induced by trauma or chemical burns

Table 3 Diagnostic criteria for non-inflammatory degenerative keratopathy

Keratoconus
Simple or secondary endothelial atrophy
Corneal dystrophies (i.e. Fuchs' dystrophy)
Excluded: Scarring induced by trauma, chemical burns or unknown factors

Group 3 (negative control), degenerative keratopathy. Cases of established degenerative corneal disease with no clinical evidence of an active or previous corneal inflammatory episode (Table 3).

Since no generally accepted set of criteria exists for an unequivocal differentiation between each and every clinical entity, the distinguishing features summarized in Tables 1, 2 and 3 are based upon our own clinical experience. Accordingly, clinically typical cases of herpetic keratitis (group 1) are separated from the broad experimental category (group 2) embracing all other forms of inflammatory keratitis, including possibly atypical HSK, and from the negative control group, which includes all non-inflammatory forms of keratopathy (group 3). For a differentiation on this basis, all patients with corneal disease attributable to trauma, chemical burning or an unknown cause had to be excluded [15].

Collection of corneal buttons

Excised corneal buttons were tranisected through the scarred region, immediately fixed in buffered 4% formaldehyde solution for 24 h and then embedded in paraffin. Tissue sections 30 µm thick were prepared and used for DNA extraction and amplification, as described in detail elsewhere [14]. Briefly, DNA was liberated by digesting the tissue in 100 µl of proteinase K buffer for 30 min at 60°C and for 10 min at 95°C [13, 18]. After centrifugation, 5 µl of the supernatant were withdrawn and either used immediately for the amplification assay or stored at –20°C for future analysis.

From the second half of the scarred corneal button, an approximately 100-µg sample was excised and incubated in 200 µl of proteinase K buffer as described above. After centrifugation (10,000 *g* for 10 min), the supernatant was withdrawn and either used immediately for DNA amplification or stored at –20°C for future analysis [13].

Methods for the detection of HSV-1 DNA or antigens

DNA amplification and hybridization. The amplification procedure has been fully described elsewhere [13]; only the pertinent details are given here. DNA amplification was run under standard conditions. Primers were directed against the glycoprotein D gene (amplification fragment length: 221 base pairs [1]) and the thymidine kinase gene (amplification fragment length: 110 base pairs [23]). Carryover was prevented by a pre-amplification cleavage with uracil N-glycosylase [22] and by incorporating uracil triphosphates into the amplificates. All buffers and media used were routinely run as internal negative controls to exclude the possibility of viral contamination. Internal controls for specificity included culture supernatants from cells infected with HSV-1, HSV-2, varicella-zoster virus or cytomegalovirus. In each amplification experiment, positive internal controls containing 10 ng of an HSV-1 DNA equivalent, consisting of approximately 10 viral copies (fragment length: 221 base pairs), were also included. Each positive result was confirmed by an independent control amplification. Negative samples were sporadically spiked with minute amounts of control HSV-1 DNA and run in separate PCRs to detect false negatives attributable to the presence of inhibitory factors. A tissue was deemed to harbour HSV-1 DNA if this was amplified from one of the two samples of fresh or paraffinized tissue.

Immunohistochemistry. Sections of paraffin-embedded corneal tissue 6 µm thick were transferred to SuperFrost slides (Wetzel Glasses, Mainz, Germany), dewaxed and rehydrated. They were then incubated at ambient temperature with primary rabbit-anti-HSV-1 (B 0114, DAKO Diagnostics Ltd., Cambridgeshire, UK; diluted 1:200 in Tris-chloride buffer, pH 7.2), the antibody then being detected according to the streptavidin/biotin method (in compliance with the manufacturer's instructions; LSAB-2 kit, DAKO Diagnostics, Cambridgeshire, UK).

Positive and negative internal controls consisted of a pair of donor corneas that failed to meet our transplantation criteria. One of the corneas, maintained in minimal essential medium, served as the negative control, whilst its partner, infected in vitro with a clinical HSV-1 isolate, served as the positive control. These controls were always run in parallel so as to be able to define the signal/noise amplitude. The evaluation was conducted according to a standard protocol, the investigator being kept in ignorance of information pertaining to patients. To assess specificity, we also evaluated the distribution and cell-association of positive signals. In the event of high background staining or the inability to interpret a given result, a second experiment was run using a higher dilution of the antibody (1:1000 instead of 1:200).

Statistical analysis

Data were analysed using the "Statistica for Windows" program, version 5.1 (StatSoft, Inc., Tulsa, Okla., USA), the χ^2 test being employed for comparisons between two groups and the maximum-likelihood χ^2 test for those involving all three. Statistical significance was set at a p value of <0.05.

Results

Immunohistological staining of corneal explant tissue revealed the presence of HSV-1 antigens in 8 (11%) of the 72 buttons excised from patients with non-HSK, in 16 (31%) of the 51 buttons derived from individuals with HSK (χ^2 test: p=0.01), and in 1 (3%) of the 30 taken from subjects with non-inflammatory degenerative kera-

Table 4 Analysis of corneal explant buttons

Detection of	HSK (n=51)	Non-HSK (n=72)	p value HSK/ non-HSK	Degenerative keratopathy (n=30)
Antigen (Ag)	16/51 (31%)	8/72 (11%)	0.01	1/30 (3%)
DNA	11/51 (22%)	16/72 (22%)	0.97	1/30(3%)
Ag and DNA	8/51 (16%)	6/72 (8%)	0.32	1/30 (3%)
Ag and/or DNA*	19/51 (37%)	18/72 (25%)	0.20	2/30 (7%)

*p=0.01 (HSK vs non-HSK vs degenerative keratopathy)

topathy. The analysis for HSV-1 DNA yielded corresponding numbers of 16/72 (22%), 11/51 (22%; χ^2 test: p=0.97) and 1/30 (3%; χ^2 test: p=0.06).

Taking these findings together, HSV-1 antigens and or DNA were detected in 18 (25%) of the 72 buttons excised from patients with non-HSK, in 19 (37%) of the 51 buttons derived from individuals with HSK (χ^2 test: p=0.20), and in 2 (7%) of the 30 buttons taken from subjects with non-inflammatory degenerative keratopathy (χ^2 test: p=0.01) (Table 4).

Discussion

In this study, we evaluated corneal explant tissue immunohistochemically for the presence of HSV-1 antigens (against glycoprotein D) and for that of HSV-1 DNA by amplification and hybridization. By these means, we attempted to define the role of HSV-1 in the pathogenesis of non-HSK at the time of corneal transplantation.

Clinical signs and symptoms represent the sole established basis for diagnosing HSK, which, for some elusive reason, cannot be confirmed by laboratory testing in the majority of cases, despite important therapeutic consequences. Although the clinical signs and symptoms are deemed to be characteristic (Table 1), our data indicate that they are unable to differentiate clearly between HSK and non-HSK at the time of corneal transplantation. There exists a substantial body of evidence indicating that many cases of HSK are either overlooked or diagnosed late, which is a disquieting circumstance. In concrete terms, the fact that HSV-1 was detected in only 37% of the samples derived from patients with active HSK implies that the detection sensitivity was likewise 37%. On this basis, the 25% detection level revealed for the non-HSK group represents an underestimation of the HSV-1 contribution, the truer value lying around 60%.

It should also be borne in mind that the majority of patients had undergone long-term, multidrug anti-inflammatory treatment for previous and current disease states prior to corneal surgery. This circumstance will inevitably modify the clinical as well as the histological picture of the disease and might thereby influence the interpretation of the clinical situation and reduce the predictive value of the immunohistochemical evaluation. Indeed, the immunohistochemical analysis confirmed the presence of HSV-1 in less than one third of the cases with HSK. In tissue evincing little to moderate inflammation, the HSV-1 antigen is readily detected, but when chronic stromal infiltration and vascularization are manifest, the signal is often masked by intense non-specific background straining. Although the severe inflammatory reaction may be partially attributable to previous treatment, the immunohistochemical mapping of HSV-1 is nonetheless known to yield highly variable results [2, 16].

In cases of active herpetic uveitis and keratouveitis, our own aqueous humour analyses have led us to the realization that antigen degradation (possibly by mononuclear cells [10, 30]) or neutralization is responsible for a marked attenuation in detection sensitivity with increasing time after the onset of inflammation [37]. The existence of such a relationship has been demonstrated experimentally [2], but not in a clinical setting. HSV-1 DNA is known to be more resistant to enzymatic degradation than HSV-1 antigens in the corneal environment [2], which explains the twofold higher detection rate for HSV-1 DNA.

Although a positive amplification signal proves the local presence of HSV-1, it does not necessarily follow that the virus contributes to the clinical disease [29]. Added to this uncertainty, we have no conception of how the presence of viral DNA in clinically healthy corneal tissue [5, 8, 14, 27, 32] is to be understood. The possibility of contamination has, of course, to be considered. But several independent laboratories, each with a long-standing reputation in DNA amplification, and each taking the utmost precautions to prevent the carryover of amplification products, have obtained identical results in this respect [6, 14, 24, 42]. We are thus forced to conclude that the presence of HSV-1 DNA in the human cornea may be harmless or even physiological. However, the mechanism underlying its tolerance, as well as its possible physiological function, have yet to be defined, and the possibility of its playing a hazardous role after the transplantation of infected corneal tissue also remains to be clarified [5, 36, 41].

In conclusion, a high proportion of cases with non-herpetic or clinically atypical stromal keratitis (non-HSK group) harboured HSV-1 antigens or DNA, the clinical relevance of which remains to be clarified. On the other hand, the applied case-definition criteria for HSK, although generally acceptable, are perhaps too stringent, in

that a substantial number of individuals with ambiguous clinical signs and symptoms are excluded and assigned to the non-HSK group. Therefore in cases of non-HSK which do not respond in the expected manner to treatment for a non-herpetic condition, it may be expedient to instigate supplementary anti-herpetic and immunomodulatory therapy.

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