Suppression of thrombospondin 1 and 2 production by herpes simplex virus 1 infection in cultured keratocytes

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Purpose: Stromal vascularization is a frequent occurrence in herpes simplex keratitis (HSK) and carries a poor prognosis for penetrating keratoplasty. The pathogenesis may involve disruption of the normal equilibrium between angiogenic and anti-angiogenic factors in and around the cornea. Thrombospondin (TSP) 1 and 2 are multifunctional matricellular glycoproteins with potent anti-angiogenic properties and are expressed by human keratocytes in a stromal wound repair model. We hypothesize that the synthesis of these anti-angiogenic proteins by keratocytes is inhibited by HSV1 and that such a mechanism may contribute to stromal vascularization in HSK.

Methods: Nonconfluent monolayers of human keratocytes were infected with HSV1 at a multiplicity of infection of 5 virus particles/keratocyte. Expression of TSP1 and TSP2 was determined by immunohistochemistry and SDS-polyacrylamide gel electrophoresis at 0, 2, 4, 6, 8, 24, 48, and 72 h after infection (ai). Expression of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) served as a control. Expression of immediate early and late viral proteins was also determined. Protein expression was quantified by densitometric analysis of the immunoblot bands.

Results: Human keratocytes supported the growth of HSV1 at all times ai. TSP1 and TSP2 were downregulated as early as 4 h ai to a 50% reduction by 8 h (p<0.002), and were absent from 24 h ai (p<0.001). There was no change in the level of expression of GAPDH throughout the duration of the experiment. Immediate early viral proteins (HSV1:ICP27) could be detected from 6 h ai reaching maximum intensity 24 h ai and late proteins (HSV1:1gD) were expressed from 24 h.

Conclusions: The synthesis of TSP1 and TSP2 is selectively downregulated by HSV1 infection in human keratocytes. Addition of these proteins or their angi-active peptides in early stage HSK therapy may be an important adjuvant in controlling HSV1 induced corneal vascularization.

Herpes simplex keratitis (HSK), with a prevalence of 20.7x10⁶/year, is the most common infective cause of unilateral blindness and the main infectious indication for penetrating keratoplasty in many developed countries [1,2]. Stromal vascularization is a frequent occurrence in chronic HSK [3-5] and corneal transplantation carries a high risk of failure in these cases since the angiogenic and immunologic privilege of the cornea is lost [6,7]. Although the pathogenesis of herpes simplex virus 1 (HSV1) induced corneal vascularization is not clearly defined, procedures that abrogate or minimize angiogenesis may diminish the severity of HSK [7-10].

It is thought that disruption of the normal equilibrium between the angiogenic and anti-angiogenic stimuli may be responsible for stromal vascularization in HSK [11,12]. Unlike other viruses, HSV does not specifically encode for angiogenic proteins [8]. HSV has however been shown to selectively suppress matrix protein synthesis including that of fibronectin, collagen I and III, and thrombospondin in smooth muscle and vascular endothelial cells [13-16].

Thrombospondin (TSP) is a family of at least 5 secreted glycoproteins, the first two of which (TSP1 and TSP2), are trimeric molecules with individual chain sizes of 110-180 kDa. They have potent anti-angiogenic properties [17-23]. We have shown previously that TSP1 and TSP2 are expressed by keratocytes in an in vitro stromal wound repair model [24,25]. Moreover, keratocyte synthesis of TSP1 is up regulated following various stromal insults in vivo [26,27]. Hence, TSP1 and TSP2 are well placed to play a role in regulating corneal angiogenesis during corneal disease [24,25,28]. We hypothesize that the synthesis of these anti-angiogenic proteins by keratocytes may be inhibited by HSV1 and that such a mechanism might contribute to corneal stromal vascularization in HSK. We therefore examined the effect of HSV1 infection on the expression of TSP1 and TSP2 by human keratocytes.

METHODS

Human keratocyte cell culture: Cell cultures of human corneal keratocytes were established from donor corneas, obtained locally, not suitable for transplant (St. Paul’s Eye Unit, Royal Liverpool University Hospital, Liverpool, United Kingdom) in accordance with the local research ethics committee. Keratocytes were harvested as previously reported by our group [26]. Keratocytes were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium: Ham’s nutrient mixture F-12 (DMEM:F-12) supplemented with 10% fetal calf serum and 1% each of glutamine, penicillin, streptomycin, and fungizone. Primary cultures were kept in 5% CO₂ in humidi-
fied air at 37 °C. During passage of primary cultures, keratocytes were detached using 0.1% trypsin/EDTA solution. Medium was changed every three days. Nonconfluent keratocytes between passages five and eight were used for the experiments.

**Virus preparation:** Wild type HSV1 (strain H 239) isolated from a patient with Herpes labialis was propagated on Vero cells (Department of Medical Microbiology, University of Liverpool). Infected Vero cell monolayers were harvested when viral cytopathic effects were present on all cells. The cells were then freeze-thawed three times, homogenized, and centrifuged at 1,500x g to remove cell debris. The purified virus was re-suspended in growth medium, divided into aliquots, and stored at -80 °C until use. Virus titer was evaluated by a standard plaque assay and a stock suspension of 10^7 plaque forming units/ml (pfu/ml) was used for inoculation [13-16].

**HSV1 infections:** Human keratocytes were seeded onto four and six well tissue culture plastic slides (Lab Teks; Nalge NUNC Inc., Naperville, IL) at a density of 4x10^4 cells per well. Monolayers were washed with phosphate buffered saline (PBS) and infected with a multiplicity of infection (MOI) of 5 pfu/cell in accordance with previous studies [13-16]. Unabsorbed virus particles were removed 20 min after inoculation and cultures were rinsed three times with PBS. Cultures were then re-fed with growth medium and incubated in a 5% CO2 humidified atmosphere at 37 °C. The time of addition of virus inoculum was taken as time point zero. Monolayers were examined under the phase contrast microscope for cytopathological changes at different time intervals. For the purpose of this study monolayers were examined at 2, 4, 6, 8, 24, 48, and 72 h after infection (ai) because the cytopathic effects of HSV are manifest in this time period [13-16]. Monolayers sham treated with equal volumes of uninfected cell growth medium served as controls. Evidence of infection was obtained by assessing the degree of cytopathic effects and the expression of viral proteins in keratocytes.

**Analysis of proteins: western blot analysis:** Proteins were extracted from kerocyte monolayers in lysis buffer (0.12 M β-mercaptoethanol, 40 mM Tris, 10% glycerol, 1% SDS, 0.01% bromophenol blue) after two washes with PBS 2, 4, 6, 8, 24, 48, and 72 h ai. Equal amounts of protein samples (containing approximately 1x10^4 cells), as determined by BCA protein assay, were boiled for 5 min and centrifuged before loading. Polyacrylamide gel electrophoresis (PAGE) was performed on a 10% polyacrylamide SDS Tris-glycine gel in 0.025 M Tris, 0.25 M glycine and 0.1% SDS. Prestained molecular weight markers (Bio-Rad Laboratories Inc., Hercules, CA) were used as standards. Resolved proteins were transferred onto a nitrocellulose membrane (N-2639; Sigma-Aldrich Corp., St Louis, MO). After SDS-PAGE the gels were stained with 5% Coomassie brilliant blue and destained in 7% acetic acid and 15% methanol mixture to check for completion of protein transfer. Transfer was accomplished by subjecting the gels to 100 amps for 2.5 h at 4 °C. After electroblotting the nitrocellulose paper was washed twice with wash buffer (0.2 M Tris NaCl pH 7.6, 0.1% Tween-20) and treated with blocking buffer (5% nonfat dry milk in wash buffer) for 1 h at room temperature with gentle agitation. It was then incubated with the appropriate primary antibody for 1 h at room temperature, washed three times in wash buffer and incubated in an appropriate horse raddish peroxidase (HRP) conjugated secondary antibody for 1 h at room temperature (A 4416 and A 5420; Sigma-Aldrich Inc.). Immunoblot detection was by the enhanced chemiluminescence technique (ECL reagents-RPN 2209; Amersham Biosciences, Buckinghamshire, UK). The membranes were exposed to the reagents for 1 min and then exposed to the X-ray film for 5, 15, and 30 s and developed.

Relative protein abundance was determined by densitometric analysis, in which quantifying the signals on the X-ray film were quantified using MCID basics software (Interfocus Limited, Cambridge, UK). For the purpose of this study the antibodies used were listed in Table 1. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis, was employed as a reference protein for the densitometric analyses.

**Analysis of proteins: immunohistochemical staining:** Infected monolayers in 4 well slides (4x10^4 cells) were washed

### Table 1. Source and concentration of antibodies used in protein analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
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<tr>
<td>TSP1 (N-20)</td>
<td>sc-12312</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>TSP2 (N-20)</td>
<td>sc-7655</td>
<td>1:100</td>
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<tr>
<td></td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>GAPDH-6C5</td>
<td>AB-8245</td>
<td>1:2000</td>
</tr>
<tr>
<td></td>
<td>ABcam</td>
<td></td>
</tr>
<tr>
<td>HSV1 ICP27 (vp-20)</td>
<td>sc-17544</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Santa Cruz</td>
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<tr>
<td>HSV1 gD (vn-20)</td>
<td>sc-17540</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td>Santa Cruz</td>
<td></td>
</tr>
<tr>
<td>Anti-goat IgG (HRP conjugate)</td>
<td>A4416</td>
<td>1:1000</td>
</tr>
<tr>
<td></td>
<td>Sigma</td>
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</tr>
<tr>
<td>Anti-mouse IgG (HRP conjugate)</td>
<td>A5420</td>
<td>1:2000</td>
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TSP1 and TSP2 protein expression was determined in sham infected and HSV1 infected human keratocyte cultures by immunohistochemical staining and western blotting. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis, is widely expressed by all living cells. Its expression by mock infected and HSV1 infected keratocytes was used as a control for western blot analysis. HSV1 infected cell protein (HSV1 ICP27) was used to determine the expression of immediate early viral proteins and HSV1 glycoprotein D (HSV1 gD) was used for the detection of late viral proteins. The HRP conjugated antibodies were the secondary antibodies used in the immunohistochemical procedures.
twice with PBS and fixed in methanol at -20 °C for 10 min at different time intervals ai. These preparations were immunohistochemically stained at room temperature. Briefly, monolayers were incubated in 1% H₂O₂, washed with wash buffer (0.05 M Tris-HCL, pH 7.6 containing 0.15 M NaCl and 0.05% Tween-20 [S3006; Dako Cytomation, Carpenteria, CA]) and nonspecific protein binding blocked with 1:5 rabbit serum (S-5000; Vector, Burlingame, CA). The cells were then incubated with polyclonal goat primary antibodies raised against synthetic peptides from regions of TSP1 and TSP2 (sc-12312 and sc-7655; Santa Cruz Technology, Santa Cruz, CA) diluted 1:100 (2 µg/ml) in wash buffer (WB) with 1% rabbit serum for 50 min. Following three washes with WB, cells were incubated with biotinylated anti-goat secondary antibody (Streptavidin-Biotin Complex, Vector, Burlingame, CA).

Figure 1. Immunohistochemical staining. **A,B:** Nonconfluent cultured keratocytes labeled for TSP1 (**A**) and TSP2 (**B**) before reaching confluence (DAB staining). **C:** TSP1 immunoreactivity was localized in a diffuse granular perinuclear pattern with peripheral punctuate staining. **D:** No staining could be visualized in the control procedures in which primary antibody was replaced by IgG fragments. **E:** Keratocytes have a rounded configuration at 72 h ai with HSV1 (hematoxylin & eosin). **F:** A clear reduction in the protein signal (compared to **B**) is seen for TSP2 4 h ai (DAB staining). The scale bars represent 54 µm in **A,B,D,F** and 50 µm in **C,E**.
antibody (BA 5000; Vector) diluted 1:200 for 30 min. They were then washed with WB three times and incubated with (HRP) conjugated streptavidin (SA 5004; Vector) diluted 1:500 for 30 min. Following three more washes with WB, immunoreactive sites were visualized brown with diaminobenzidine (DAB; Dako Cytomation Envision kit). Slides were mounted in aqueous mounting medium (Dako Cytomation) for light microscopy. Negative controls were performed by replacing the primary antibody with preimmune goat IgG fragments (sc-2028; Santa Cruz) while keeping all other steps the same.

Statistics: Samples were compared using the Student’s t-test. A p < 0.01 was taken as statistically significant.

RESULTS

Expression of TSP1 and TSP2 in uninfected cultured human keratocytes: Nonconfluent cultures of keratocytes were immunoreactive for TSP1 and TSP2. Immunoreactivity for each protein was localized in a diffuse granular perinuclear pattern and a punctuate staining in the cell periphery. No staining was seen in preparations in which the primary antibodies were replaced with preimmune IgG (Figure 1A-D). Moreover, TSP1 and TSP2 proteins were visualized as 180 kDa bands in immunoblot analysis by reducing SDS-PAGE in cultured keratocytes (Figure 2A-C).

Evidence of HSV1 infection in keratocytes: Human keratocytes supported the growth of HSV1 at all times ai. Cells in infected cultures appeared refractile, rounded, and retracted leading to increased intercellular spaces as early as 4 h ai and by 72 h some cells had detached from the surface of the flask (Figure 1E). No such cells were detected in sham infected cultures. Immediate early viral proteins (HSV1:ICP27) were first detected by western blotting at 6 h ai (Figure 2D,E) and reached maximum intensity at 24 h ai. Late proteins (HSV1:glycoprotein D) could be detected at 24 h ai reaching maximum intensity at 72 h (data not shown). No viral proteins were detected in sham infected cultures.

Effect of HSV1 infection on TSP1 and TSP2 expression: Protein expression of TSP1 and TSP2 was measured by densitometric analysis of autoradiographs from reducing SDS-PAGE analysis at different times ai compared to sham infected cultures. There was a 50% reduction in the expression of each protein by 8 h ai (p<0.002). There was complete absence of detectable TSP1 and TSP2 by 24 h and all later times ai (p<0.001, Figure 2A,B). There was no change in the expression of TSP1 and TSP2 in sham infected cultures at any time ai (Figure 2C). Suppression of these proteins commenced before the expression of HSV1 immediate early proteins (6 h). Clear suppression of TSP1 and TSP2 protein expression was visualized by immunohistochemical staining as early as 4 h ai (Figure 1F).

Effect of HSV1 infection on GAPDH expression: GAPDH was expressed by keratocytes at all times and visualized as a 36 KDa band following immunoblot analysis (Figure 2D). There was no change in the expression of protein at any time.

Figure 2. Western blot analysis. Protein expression of TSP1, TSP2, TSP1 (control), GAPDH, and HSV1:ICP27 at different time intervals ai. Densitometric analysis revealed there was a 50% reduction in the signal intensity of TSP1 (A) and TSP2 (B) by 8 h ai, with a complete absence by 24 h ai. There was no change in the expression of TSP1 in sham infected cultures (C). There was no change in the expression of GAPDH at any time ai (D). Immediate early viral proteins (HSV1:ICP27) were expressed by 6 h, reaching maximum intensity at 24 h ai (E).
DISCUSSION

We have successfully established an in vitro system to study the early molecular changes after HSV1 infection in human keratocytes. Our observations show that synthesis of TSP1 and TSP2 is downregulated by HSV1 infection of human keratocytes in vitro and these proteins cannot be demonstrated by 24 h ai. This response is selective for TSP1 and TSP2 in that it is not observed for GAPDH. Moreover there is a concomitant increase in the expression of viral proteins during the same period that TSP1 and TSP2 expression decreases. These results suggest a possible role of TSP1 and TSP2 in the pathophysiology of HSV1 stromal keratitis.

TSP1 and TSP2 are expressed in normal human and bovine corneal endothelium, and in a less consistent pattern, the basal corneal epithelium. The corneal stroma and stromal cells (keratocytes) however are normally devoid of these proteins [24], although keratocytes contain TSP1 (but not TSP2) mRNA [26]. Following stromal injury, keratocytes have the ability to synthesize TSP1, TSP2, and thrombospondin 3 [25,26]. Thrombospondin 3 is a pentameric thrombospondin and has not been shown to be anti-angiogenic. Conversely, TSP1 was the first naturally occurring protein shown to be anti-angiogenic [29] and the angiostatic effects of TSP1 and TSP2 have been well documented in numerous in vivo and in vitro studies [17-23]. TSP1 inhibits migration and proliferation of vascular endothelial cells, induces apoptosis, and blocks capillary tube formation [19]. TSP1 and TSP2 can bind and inhibit the activity of potent pro-angiogenic factors such as fibrosing growth factor (bFGF), vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP) 9, and MMP2 in the cornea and chick chorioallantoic membrane assays [17,19,20,23,29,30].

Cursiefen et al. [28] have demonstrated in a murine model that developmental corneal avascularity is redundantly regulated, but induced adult/postnatal corneal vascularity is dependent on TSP1 and TSP2 and TSP1 playing the major role. They concluded that if postnatal angiogenic stimuli exceed the threshold of protection provided by other inhibitors, the absence of TSP1 and TSP2 permits induced corneal neovascularization to proceed. Our results are consistent with this finding. We propose that TSP1 and TSP2 appear to be suppressed early and persistently after HSV1 infection and their loss may play a part in HSV1 induced stromal vascularization.

The mechanism behind HSV1 induced angiogenesis is poorly understood. Unlike other viruses HSV1 does not encode for angiogenic proteins [8], which suggests that infection alters the balance between pro and anti-angiogenic factors in tissues. A few early reports have shown a generalized reduction in cellular proteins like fibronectin, procollagen, and thrombospondin by HSV1 infection in smooth muscle and vascular endothelial cells [13-16]. The degree of suppression was dependent on the dose of the virus inoculum and type of protein [13-15]. Human cytomegalovirus (CMV), a member of the herpes virus family, has the potential to selectively downregulate or upregulate cellular mRNAs in vitro and can reduce TSP1 mRNA by a factor of 21 and TSP2 by a factor of 13, 24 h ai [31]. CMV infection decreases the expression of TSP1 and TSP2 in human retinal glial cells, and a possible role of TSP1 and TSP2 in optic disc neovascularization in CMV retinitis has been suggested [32].

The selectivity of the effects of HSV1 on TSP1 and TSP2 production is further highlighted by the observation that HSV1 upregulates angiogenic factors such as VEGF [7,8] and MMP 9 [7] by several cell types [7-10]. Given that TSP1 and TSP2 can inhibit VEGF function [22] it seems that the loss of TSP1 and TSP2 coupled with the increase in VEGF may produce a stromal environment that is highly favorable to blood vessel ingrowth.

Conical neovascularization is an early and important event in the development of stromal HSK and angiostatic factors have a beneficial effect on the severity of infection [8-10]. Treatment with angiogenesis inhibitors from the outset of infection significantly reduces the severity of HSV stromal keratitis and vascularization [9]. Our results indicate that addition of TSP1 and TSP2, or their angio-active elements [20,21], in the early stage of the disease may enhance existing or provide alternative anti-angiogenic therapy in HSK and other conditions that lead to corneal stromal vascularization. Indeed, anti-angiogenic peptide fragments of TSP1 and TSP2 are already being involved in clinical trials for the control of tumor angiogenesis [30,33,34] and the findings of the present investigation suggest that such trials be extended to encompass corneal stromal vascular disease.

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REFERENCES

9. Zheng M, Schwartz MA, Lee S, Kumaraguru U, Rouse BT, Con-