Herpes simplex virus type 1 infection has two separate modes of spread in three-dimensional keratinocyte culture

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This study describes the outcome of herpes simplex virus type 1 (HSV-1) infection in an organotypic raft culture of spontaneously immortalized HaCat keratinocytes and human fibroblasts, as related to the virus load and epithelial stratification and differentiation. In this model, a confluent monolayer of HaCat keratinocytes was formed 60 h after seeding. Inoculation of HSV-1 before induction of differentiation by lifting of the culture to the air–liquid interface always resulted in a productive infection, but the virus yield was highest when the inoculation took place 72 h after seeding. Even at 0–1 p.f.u. per culture, the HaCat cultures became HSV positive. Infection of the full-thickness epithelium at 5 p.f.u. per culture resulted in a productive infection of the whole epithelium. The HaCat cells were about 10 times more sensitive to HSV-1 infection than the Vero cells in which the virus stocks were titrated. The raft cultures infected 30 min after lifting were negative by HSV-1 culture, and no HSV-1 antigen was detected by immunocytochemistry. PCR showed the presence of HSV-1 DNA and in situ hybridization showed reactivity with a latency-associated RNA probe, indicating the presence of a non-productive infection. Two different patterns of virus spread in epithelia were found: (i) lateral spread through the superficial layers of the epithelium and (ii) a demarcated infection throughout the whole thickness of the epithelium at the margins of the culture.

Introduction

Organotypic three-dimensional (raft) culture of keratinocytes provides a tissue culture model with characteristics of differentiated epithelium (Asselineau & Prunieras, 1984). Previously, raft cultures have been successfully applied to the culture of human papillomaviruses (Meyers & Laimins, 1994). We were the first to demonstrate the applicability of this organotypic tissue culture to the study of herpes simplex virus type 1 (HSV-1) infection (Syrjänen et al., 1996). A similar HSV-1 culture system has also been described by Visalli et al. (1997).

Productive HSV infection involves the coordinately regulated and sequentially ordered expression of defined classes of viral genes, the α, β and γ genes (Hones & Roizman, 1974, 1975). Lytically HSV-infected cells are subject to structural alterations, accompanied by microscopically visible cytopathic effects, including reticular degeneration and ballooning of the cells, as well as formation of multinucleated giant cells (Roizman & Sears, 1996). In the organotypic culture of epithelial cells, lytic HSV-1 infection shows features typical of an epithelial infection in vivo (Syrjänen et al., 1996; Visalli et al., 1997). Lytic infection was always detectable throughout the whole epithelium when HSV-1 (5 p.f.u.) was inoculated on the epithelial monolayer (HaCat) at the time of confluence before lifting the cultures. Visalli et al. (1997) applied HSV-1 to the top of a cornified full-thickness epithelium (epithelial cells from foreskin and ectocervix) grown for 8 days. They reported that HSV-1 could penetrate into the basal cells and initiate replication there. Some areas were infected, whereas some fields of the culture remained apparently normal by light microscopy. The limitation of virus spread appeared to be dependent on the HSV strain or mutant used (Visalli et al., 1997).

HSV establishes a latent infection in sensory neurons (Roizman & Sears, 1996). In the numerous in vitro models established in different cell types (Levine et al., 1980; Rapp, 1984; Nilheden et al., 1985; Biswal et al., 1988), replication of HSV is restricted by various treatments of the cell cultures, having little resemblance to the early events of a latent
infection in the sensory neurons of animals (Roizman & Sears, 1996). In our recent study, we also described a form of HSV-1 infection without cytopathic effects in epithelial raft culture. The presence of HSV-1 was demonstrated by in situ detection of latency-associated RNA (LAT RNA) (Stevens et al., 1987; Wagner et al., 1988) and by PCR for HSV-1 DNA (Syriänen et al., 1996). The appearance of this type of infection was dependent on the timing of virus application relative to epithelial differentiation, requiring virus infection early after induction of stratification (Syriänen et al., 1996). Epithelial latency of HSV in humans has been suggested previously on the basis of indirect evidence in an HSV reactivation study (Spruance et al., 1991). Persistent HSV-1 infection in murine non-neuronal cells has also been suggested (Maggis et al., 1998). The non-productive form of HSV-1 infection (Syriänen et al., 1996) would support the concepts of epithelial latency or persistence.

In the present report, we describe the spread of HSV-1 infection in an organotypic culture and describe the establishment of HSV-1 infection as a function of the virus load, growth properties of the epithelium and the level of epithelial differentiation.

Methods

- Virus. HSV-1 strain F, obtained from the ATCC (Manassas, VA, USA) was used throughout the study. The virus stock was prepared in human foreskin fibroblasts (HFF) as described previously (Syriänen et al., 1996) and stored at −70 °C at a concentration of 3·6 × 10⁶ p.f.u./ml (Vero cell infectious units).

- Organotypic cell culture. HaCat cells (Boukamp et al., 1988) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% non-essential amino acids, 2 mM l-glutamine, 50 µg/ml streptomycin, 100 U/ml penicillin and 10% foetal calf serum (Gibco BRL). Human skin fibroblasts were prepared as described previously (Syriänen et al., 1996) and grown in supplemented DMEM.

- The fibroblast–collagen gel was prepared as described previously (Syriänen et al., 1996). Briefly, human skin fibroblasts were suspended in a solution of Vitrogen 100 collagen (Celtrix Pharmaceuticals) in DMEM at pH 7·4 at a density of 280 000 cells per 0·7 ml and plated on 24-well tissue culture dishes (Costar). The cells were maintained at 37 °C in Green’s medium (DMEM–HamF12: 3:1 medium containing 10% foetal calf serum, 4 mM glutamine, 5 µg/ml insulin (Sigma), 0·18 mM adenine (Sigma), 0·4 µg/ml hydrocortisone (Sigma), 0·1 nM cholera toxin (Sigma) and 5 ng/ml epidermal growth factor (Boehringer Mannheim)). Ascorbic acid was added to the medium just before use to a final concentration of 50 µg/ml.

For organotypic cultures, HaCat cells were added onto fibroblast–collagen gels at a density of 200 000 cells per well. The cultures were grown submerged in Green’s medium for 52–78 h as indicated and lifted to the air–liquid interface by using a stainless steel grid support. The cultures were infected with HSV-1 at time-points of 24 h before lifting or 25–6 h after lifting as described. The amount of infectious virus varied between 0·1 and 10³ p.f.u. per culture, expressed as Vero cell infectious units. The cultures were harvested 1 week post-infection (p.i.). The cultures were collected at the times indicated and divided into equal parts for HSV-1 culture, microscopic analyses and DNA extraction. For microscopy and immunohistochemistry, the samples were fixed in buffered 10% formalin for 24 h.

- PCR for HSV-1 DNA. The PCR test for HSV-1 DNA was carried out as described previously (Syriänen et al., 1996) with gD-1 primers described by Aurelius et al. (1991). The specimens were DNA extracts from frozen cultures. Positive controls were HSV-1 (F) virus, 1 p.f.u. from a 10⁶ p.f.u./ml stock per reaction. The working spaces and devices were designed to minimize contamination.

- In situ hybridization. In situ hybridizations were carried out according to our previous protocol (Syriänen et al., 1996) with digoxigenin (DIG)-labelled single-stranded RNA probes. The LAT RNA probe contained sequences from the 0·5 kb HpaII–SalI subfragment of HSV-1 (F) BamHI fragment B. The DIG RNA probe for zTIF (VP16) mRNA was transcribed from plasmid pBR3717 (McKnight et al., 1987; a gift from Bernard Roizman, University of Chicago, IL, USA) by using T7 polymerase.

- Immunohistochemistry. The presence of HSV-1 antigen was demonstrated in paraffin sections of the epithelial cultures by immunohistochemistry with a polyclonal rabbit antibody against HSV-1 (BioGenex Laboratories) diluted 1:100. The sections were pretreated in a microwave oven with 10 mM citric acid, pH 6·0 for 5 min. Immunohistochemistry was performed with a staining automate (Dako TechMate 500) according to the manufacturer’s instructions. Formalin-fixed sections from biopsy samples taken from labial herpes were used as positive controls. In the negative controls, the primary antibody was omitted from the reaction mixture. Antibodies against ICPO and thymidine kinase of HSV-1 were kindly provided by Bernard Roizman (University of Chicago).

- Detection of infectious HSV-1. Infectious HSV-1 was detected in the culture supernatants and homogenates by a previously described immunoperoxidase system in Vero cells (Ziegler et al., 1988). Stock virus was titrated in duplicate wells in HFF, Vero and HaCat cells.

Results

HaCat cells are spontaneously transformed keratinocytes derived from the skin. In an organotypic raft culture, the HaCat keratinocytes stratify and form a differentiated epithelium 8–12 cell layers thick. In our model, only slightly para-keratinized epithelium is formed, without any orthokeratosis normally present in the skin. Thus the cultured epidermis is not as differentiated as the skin epidermis in vivo. During the first days of culture, before lifting to the air–liquid interface, keratinocytes grow horizontally as a monolayer until reaching confluence. We determined the time needed to reach confluence to understand the effect of differentiation and stratification of the keratinocytes on the outcome of HSV-1 infection.

HaCat cultures on a fibroblast–collagen layer were allowed to grow for 48–120 h, before being infected with HSV-1 while still in the culture medium. The morphological appearance of the keratinocyte layer was followed with respect to the continuity of the monolayer, because horizontal growth might affect the replication and spread of the virus within the epithelium. At 50–54 h post-seeding, the HaCat cell monolayer was 80–90% complete. At that point, the epithelial cells resembled basal cells from normal skin. The keratinocyte layer was confluent 60 h after seeding, after which they became proliferation-arrested. Morphologically, the epithelial cells were flattened and maintained the same phenotype for
Table 1. Detection of HSV-1 in HaCat keratinocyte raft cultures infected with different amounts of the virus 24 h before induction of differentiation and stratification

IHC, Immunohistochemistry. The number of HSV-1-positive parallel cultures of those tested is shown.

<table>
<thead>
<tr>
<th>Input virus (p.f.u. per culture)</th>
<th>Culture supernatants</th>
<th>Culture homogenates</th>
<th>Detection of HSV-1 by IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 days p.i. (1:50)*</td>
<td>6 days p.i. (1:250)</td>
<td>8 days p.i. (1:250)</td>
</tr>
<tr>
<td>0</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
</tr>
<tr>
<td>0·1</td>
<td>1/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>0·5</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* The dilution of the original culture supernatant or homogenate tested is shown.

Table 2. Titration of HSV-1 virus stock in HFF, B-Vero and HaCat cells

The mean number of plaques per culture is shown for each cell line and each dilution; means were determined from two parallel cultures.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dilution of virus stock</th>
<th>Calculated titre (p.f.u./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10⁻⁵</td>
<td>10⁻⁶</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFF</td>
<td>13</td>
<td>1</td>
</tr>
<tr>
<td>B-Vero</td>
<td>7·5</td>
<td>0·5</td>
</tr>
<tr>
<td>HaCat</td>
<td>82</td>
<td>8</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HFF</td>
<td>21·5</td>
<td>2</td>
</tr>
<tr>
<td>B-Vero</td>
<td>25</td>
<td>2·5</td>
</tr>
<tr>
<td>HaCat</td>
<td>133·5</td>
<td>13</td>
</tr>
</tbody>
</table>

96–120 h after inoculation. Application of HSV-1 before induction at different time-points resulted in a productive infection but the virus yield was found to be highest when infection took place at 72 h after seeding (data not shown).

Effect of virus load on the outcome of HSV-1 infection

HaCat keratinocyte cultures were infected at different m.o.i. of HSV-1 (F) in order to see whether there was a limit of input virus below which the cultures did not support productive infection. All cultures were infected similarly with HSV-1 1 day before lifting with different amounts of virus from 0·1 to 5 p.f.u. (Vero cell infectivity) per culture (Table 1). Even at 0·1 p.f.u. per culture, the HaCat culture supernatants were HSV-1-positive at 6 days p.i. and at 0·5 p.f.u. or more per culture, a strong productive infection always ensued. We determined the sensitivity of HaCat cells for HSV-1 and found that HaCat cells are about 10 times more sensitive than Vero cells (Table 2).

Infection of the full-thickness epithelium (cultured for 7 days after lifting) at 5 p.f.u. per culture resulted in a productive infection in 2/3 of the cultures, with morphological alterations in almost the entire epithelium (Table 3). When inoculation was done with 10⁵ p.f.u., large, multinucleated giant cells were seen. Infection with 10⁴ p.f.u. caused almost total lytic destruction of the epithelium. Some fibroblasts also became infected. These features were even more prominent when inoculation was done with 10⁵ p.f.u.

Effect of time of virus application on differentiation-induced cultures

Organotypic cultures of HaCat keratinocytes were infected at 0·25–6 h after lifting to the air–liquid interface, i.e. after induction of stratification and differentiation. Virus infection was studied by cultivation of the infectious virus from epithelia and supernatants, by immunohistochemical analysis, by PCR
Table 3. Detection of HSV-1 in HaCat keratinocyte raft cultures infected as full-thickness epithelium 7 days after lifting

The number of positive parallel cultures of those tested is shown.

<table>
<thead>
<tr>
<th>Input virus (p.f.u. per culture)</th>
<th>Supernatant</th>
<th>Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 days p.i. (1:50)*</td>
<td>5 days p.i. (1:50)</td>
<td>7 days p.i. (1:10)</td>
</tr>
<tr>
<td>0</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>5</td>
<td>1/3</td>
<td>2/3</td>
</tr>
<tr>
<td>(10^1)</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* The dilution used for testing is shown.

Table 4. Detection of HSV-1 at 7 days p.i. in HaCat keratinocyte cultures infected with 5 p.f.u. HSV-1 per culture at various times after induction of differentiation

Supernatants and homogenates were diluted 1:50 and 1:10, respectively. The number of positive parallel cultures of those tested is shown. IHC, Immunohistochemistry; nd, not done.

<table>
<thead>
<tr>
<th>Time of virus application (h)</th>
<th>HSV-1 culture from raft cultures</th>
<th>HSV-1 DNA PCR</th>
<th>LAT in situ hybridization</th>
<th>HSV-1 antigen detected by IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td>0.25</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3*</td>
</tr>
<tr>
<td>0.5</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>1</td>
<td>3/3</td>
<td>3/3</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>2</td>
<td>2/3</td>
<td>ND</td>
<td>ND</td>
<td>2/3*</td>
</tr>
<tr>
<td>4</td>
<td>2/3</td>
<td>ND</td>
<td>ND</td>
<td>1/3</td>
</tr>
<tr>
<td>6</td>
<td>2/3</td>
<td>ND</td>
<td>ND</td>
<td>1/3*</td>
</tr>
</tbody>
</table>

* Positive IHC staining for HSV-1 antigen was observed at the margins of the cultures.

for HSV-1 DNA and by in situ hybridization for LAT and αTIF RNA. The cultures were analysed at 7 days p.i.

By culture/immunoperoxidase detection, HSV-1 infection was detected in HaCat raft cultures infected 15 min and >60 min after lifting (Table 4). The cultures infected 30 min after lifting were negative by culture and no HSV-1 antigen was detected by immunohistochemical staining (Fig. 1a, b). An interesting staining pattern was detected in the specimens infected 60 min or more after lifting. We observed a productive infection at the margins of the cultures, where the epithelium had grown laterally beyond the fibroblast–collagen layer and reached the surface of the medium (Fig. 2). In these cultures, PCR showed the presence of HSV-1 DNA and in situ hybridization showed reactivity with the LAT RNA probe, but rarely with the probe for αTIF RNA, outside the productively infected margin area (Fig. 2b–d). The DIG-based detection system resulted in an intensive background staining of the collagen matrix. The productive infection of the margins was observed in many different experimental settings and contributed to the positive virus culture detection in specimens infected after lifting.

The spread of HSV-1 infection in epithelial cultures

In cases where HSV-1 was applied onto the epithelium 60 min or more after lifting, we observed two distinct patterns of virus spread: lateral spreading on superficial layers of the epithelium and a sharply demarcated infection throughout the thickness of the epithelium at the margins of the cultures (Fig. 3), presumably at the loci exposed to the culture medium during incubations. This infection was not limited to the basal/suprabasal cell layers; instead, the infection appeared to spread along and from the most superficial cell layers. The basal and parabasal cells remained HSV-1 antigen-negative by immunohistochemistry. Occasional infected fibroblasts were found in cultures infected with large amounts of HSV-1.
Discussion

We have previously used organotypic keratinocyte cultures to study the replication and spread of HSV-1 in epithelium (Syrjänen et al., 1996). Visalli et al. (1997) utilized a slightly different organotypic model. In both studies, a productive HSV-1 infection was established with morphological characteristics of herpes lesions in vivo.

The models presented by us (Syrjänen et al., 1996) and by Visalli et al. (1997) are not similar in all respects, which may account for the observed differences in virus spread and the outcome of the infection. We obtained HSV-1 infection throughout the whole epithelium (Syrjänen et al., 1996), whereas Visalli et al. (1997) found a productive infection in the basal and parabasal cells of their epithelium. One explanation for the contradictory results might be the inoculation time of the virus. We applied HSV-1 before lifting, when epithelial cells were confluent, while Visalli et al. (1997) inoculated HSV-1 on the surface of the full-thickness epithelium grown for 7 days. In our current model, a productive infection of the whole epithelium and even fibroblasts was produced by high input virus on the fully developed epithelium. When low titres were used to infect the epithelium 4–6 h after lifting, the infection appeared to spread along and from the most superficial cell layers.
layers. The basal and parabasal cells remained HSV-1 antigen-negative by immunohistochemistry (Fig. 3). In our model, it is unlikely that the virus penetrates the epithelium to initiate replication only in the basal cells.

According to earlier raft culture studies, fully keratinized epidermis in vitro is 10 times more permeable than human epidermis ex vivo (Regnier et al., 1990). In raft cultures, keratinocytes move from the basal layers to the cornified layer within a maximum period of 7 days, with virtually complete replacement of all viable keratinocytes in 14 days (MacCallum & Lillie, 1990). This movement might also prevent virus penetration to the basal cells, suggested by Visalli et al. (1997). The epithelium produced in our model is more immature and proliferative than the epithelium produced by Visalli et al. (1997), which might also affect the outcome of the infection.

There are essential differences in the origin of keratinocytes and fibroblasts in the two culture models. We use a spontaneously transformed HaCat keratinocyte cell line and human fibroblasts derived from the skin, whereas Visalli et al. (1997) used human foreskin and ectocervical keratinocytes as well as mouse 3T3 fibroblasts. We have recently shown that fibroblasts can modulate the phenotype of the epithelium in vitro (Atula et al., 1997). The use of human cells allows the complex interactions between keratinocytes and fibroblasts, including the actions of cytokines and growth factors, to take place. Interferon secreted by human fibroblasts is supposedly effective on human keratinocytes in our model.

As in our previous study, we obtained a form of HSV-1 infection where there was no production of infectious virus, whereas HSV-1 was detectable by PCR and RNA in situ hybridization (Syrjänen et al., 1996). In agreement with the observations of Visalli et al. (1997), there were areas of the culture that remained apparently normal, without any evidence of HSV-1 infection, as viewed by immunohistochemistry. On the other hand, we found that the margins of the epithelium were heavily infected with HSV-1 in many different experimental settings (Fig. 2a; Fig. 3a, b). This could be explained by direct contact of the keratinocytes with the culture medium as a result of an overgrowth exceeding the limits of the collagen–fibroblast bed. Horizontally growing epithelial cells are actively proliferating cells that might support virus replication. Visalli et al. (1997) reported that the infection does not proceed if the virus is distributed to the culture from below, diluted in the medium. However, in those experiments, the collagen–fibroblast layer was present between the virus and the keratinocytes. When a basal cell divides, one of the daughter cells migrates upward and starts differentiation. Thus, infected basal cells spread the infection upwards during differentiation and migration from the basal layers to the surface of the epithelium. This might result in a sharp boundary between the infected and non-infected epithelium, especially if the virus yield is high, as shown in Figs 2(a) and 3(a).

We found that the HaCat cells are about 10 times more sensitive to HSV-1 than the Vero cells used for initial virus titration (Table 2). Thus the p.f.u. values used for infection probably represent 10 times greater virus input. The non-productive infection observed therefore cannot be merely the result of an insufficient number of HSV-1 particles in the inoculum. The non-productive form of HSV-1 infection is strictly dependent on the early timing of virus application on keratinocytes after induction of differentiation. This raises the need for further analysis of the early expression of transcription factors, growth factors and other signalling molecules in the epithelium. The stratified layers of keratinocytes may express a different profile of transcription factors and growth factors than the cells shortly after induction of differentiation. The effect of HSV-1 infection at the margins of the culture blurs the opportunities to observe the non-productive infection by virus culture, since it would be positive for infectious virus even when there was a mixed productive and non-productive infection in different parts of the culture. The possibility that the LAT probe reacts just with random productively infected cells is, however, minimal, since the immunohistochemical and αTIF hybridizations for the same regions remained negative. There is some indirect support for the concept of epithelial HSV-1 latency in humans (Spruance et al., 1991) and in mice (Maggs et al., 1998). We cannot yet define our non-productive HSV-1 infection as latency, since there is no reactivation model for it at present.

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References


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