LATENT HSV-1 INFECTION CAN BE ESTABLISHED IN KERATINOCYTE CELLS FOLLOWING TREATMENT WITH MITOTIC INHIBITORS

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ABSTRACT

Herpes simplex virus type 1 (HSV-1) infection of murine keratinocyte cell lines (HEL-30 and PAM-212) treated with mitotic inhibitors leads to silencing of virus replication. At 0.1 (Multiplicity of infection) MOI, PAM-212 keratinocytes showed some cell lysis; and viral plaques were seen in HEL-30 keratinocyte cultures. PAM-212 keratinocytes, infected at 0.01 MOI, were more susceptible to the lytic effect of HSV-1 than were HEL-30 cells. In this study, 5-fluoro-2'-deoxyuridine (FUDR) treatment of both keratinocyte cell lines permitted an increase survival of HSV-1- infected keratinocytes especially for PAM-212 cell line which was further examined for the presence of sequestered virus after treatment with FUDR and infection with HSV-1. In this post-mitotic state, HSV-1-infected keratinocytes appeared to contain latent virus as suggested by the lack of virus plaques or cytopathic effect (CPE). After infection, these cells were examined for the presence of replicating HSV-1 in Vero cell overlays. Virus plaques were found suggesting that latency may have been established.

KEYWORDS: HSV-1, Mitotic inhibitors, FUDR, Taxol.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) causes serious ocular disease that can lead to blindness in both developed and underdeveloped countries[1]. In spite of a plethora of information concerning HSV-1 pathogenesis and latency, recurrent ocular infections reactivated from virus latent in trigeminal nerve neuronal cells lead to eventual loss of vision. Keratinocytes are chosen in this study because they are the primary site of infection for HSV-1 and the infection occurs via an endocytic process[2,3].

Noted that both HSV-1 and interferon-gamma (IFN-γ) rendered keratinocytes susceptible to the lytic effect of the virus and a concomitant increase in expression of suppressor of cytokine signaling-1 (SOCS-1). To render the keratinocytes susceptible to the antiviral action of interferon-gamma (IFN-γ) or its peptide mimetic and protect from HSV-1-induced lysis, the keratinocytes were pretreated with either SOCS-1 small interfering RNA (siRNA) or a peptide antagonist of SOCS-1 (pJAk2). The SOCS-1 antagonist has both an antiviral effect against HSV-1 in the keratinocyte as well as a synergistic effect on IFN-γ induction of an antiviral state and it plays an important role in the inhibition of the antiviral effect of IFN-γ in keratinocytes infected with HSV-1[3]. The present study aims to investigate whether SOCS-1 antagonists can be used to abrogate this refractoriness in vivo. Paradoxically, post-mitotic neuronal cells from the trigeminal ganglion of HSV-1-infected mice, in which HSV-1 lies latent, express high levels of SOCS-1 in response to IFN-γ[4] similar to that of keratinocytes[3]. The SOCS-1 peptide antagonist could permit reactivation of HSV-1 infection. To resolve this problem, responses of cultured neuronal cells to HSV-1 have to be determined.

The hypothesis for this study was: HSV-1 infection of murine keratinocyte cell lines (HEL-30 and PAM-212) treated with mitotic inhibitors leads to silencing of virus replication. In this post-mitotic state, the question was raised whether HSV-1 latency could be established in the treated keratinocytes. HSV-1-infected keratinocytes appeared to contain latent virus as suggested by the lack of virus plaques or cytopathic effect (CPE) after infection. To demonstrate this result, keratinocytes were examined for the presence of replicating HSV-1 in Vero cell overlays to rescue virus.

MATERIALS AND METHODS

Virus, cell lines and cell culture. HSV-1 (syn17+) (obtained from Children's Hospital Medical Center, Cincinnati, OH) was titrated in Vero cells (CCL-81, American Type Culture Collection). HEL-30 keratinocytes, derived from C3H mice, (Wright Patterson Air Force Base) and PAM-212 keratinocytes, derived from BALB/c mice, (American Type Culture Collection), were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Fisher Science, Pittsburgh, PA) supplemented with 10% bovine calf serum and 1µl/ml gentamicin sulfate solution. The cells were grown on 100 mm^2 tissue culture dishes were used to plate the cells which were incubated in a humidified incubator at 37 °C, 5% CO_2, and 95% air.

Mitotic inhibitors: 5-Fluoro-2'-deoxyuridine (FUDR) (Fisher Scientific, Pittsburgh, PA) and Paclitaxel

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(Taxol) (Fisher Science, Pittsburgh, PA) were used to induce a post mitotic state in keratinocytes. FUDR was dissolved in 2% DMEM and a stock solution of 1µg/ml was made. The stock was further diluted depending on the experiment. Paclitaxel was dissolved in DMSO as 5µg/ml stock solution.

Cytopathic effect (CPE) assay: This assay was used to determine the effect of HSV-1 treated with various concentrations of IFN- γ, FUDR, or Taxol or when FUDR or Taxol were used with IFN- γ to treat HEL-30 or PAM-212 keratinocytes. Cells were counted using a hemacytometer and plated in 96 well plates at densities of 2.0 x 10^4 to 3.0 x 10^4 per well; plates were incubated until the cells were 70-100% confluent. At that point, cells were treated with different concentrations of mitotic inhibitors. Cells were then incubated for various amounts of time depending on the experiment design before infection with virus. Virus was removed two hours later and the plates incubated for 48h in DMEM supplemented with 10% FBC. The cells were rinsed with DMEM after the end of each incubation period. Cells then were washed with phosphate-buffered saline, pH 7.4 (PBS), fixed using 10% formalin and stained with 0.05% crystal violet. Plates then were washed with H_2O and dried overnight. Plates were scanned using a ScanJet 5300C and the images were examined using NIH image J to calculate the density of cells in each well. These experiments were performed in triplicate and the results were analyzed statistically using SigmaPlot 11.2 (Systat Software, Inc., San Jose, CA)

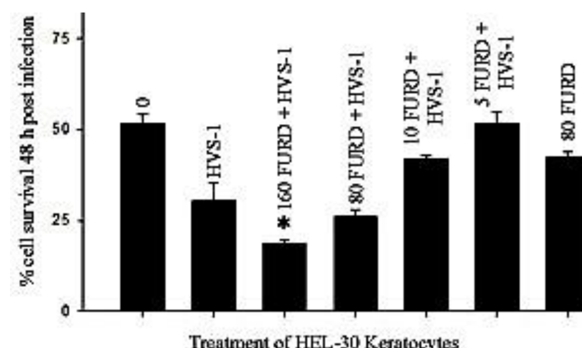
Plaque assay: After treating cells with mitotic inhibitors and infecting them with HSV-1, the virus was rescued by adding 1 X 10^5 Vero cells in DMEM containing 10% CS and the 96-well culture plates incubated for 24h at 37 °C in 5% CO_2 ; plaques were detected by methyl cellulose-containing overlay media (Fisher Science, Pittsburgh, PA). The plates were stained with 0.02% crystal violet, scanned using an HP ScanJet 5300C, and the scans examined by Image J. The results were analyzed using SigmaPlot 11.2.

RESULTS AND DISCUSSION

Selection of Dosages of Mitotic Inhibitors

In preliminary titrations (data not shown), concentrations of FUDR above 10µg/ml were toxic to the keratinocyte cell lines. Titrations of FUDR from 160 to 2.5µg/ml were added to the keratinocytes. In (figure 1), concentrations of FUDR 160µg/ml, 80µg/ml, 10µg/ml, and 5µg/ml were used. After infection with HSV-1 at an MOI of 0.1, fewer than 25% of HEL-30 cells survived these treatments. (p>0.001 by ANOVA; * p= 0.004) (figure 1).

High concentrations of FUDR induced cell death (*). For all subsequent experiments, concentrations of 5-10µg/ml of FUDR were used because each of them was non-toxic and inhibited replication of virus. Note that the 0 bar denotes the viability in culture of untreated, uninfected cells. Since Pushkarev and others found that high concentrations of paclitaxel led to cell cycle arrest in G2/M phase[5], an initial concentration of 5µg/ml was added to keratinocytes for 30 min prior to HSV-1 infection. Different HSV-1 MOI's used to infect keratinocyte cell lines.



(Figure 1) the effect of high concentrations of FUDR on HEL-30

HEL-30 and PAM-212 keratinocytes were plated in 96 well plates and infected with 0.01 or 0.1 MOI HSV-1 for 2 hours to permit adsorption of virus. The virus was then removed and fluid replaced with DMEM containing 10% CS. The keratinocyte cell lines were compared with Vero cells infected with these two MOI's. In (figure 2), note that at 0.1 MOI, each of the cell lines showed cytopathic effects; PAM-212 keratinocytes and Vero cells showed cell lysis; and viral plaques which represented in the figure as clear areas were seen in HEL-30 keratinocyte cultures. PAM-212 keratinocytes and Vero cells, infected at 0.01 MOI, were more susceptible to the lytic effect of HSV-1 than were HEL-30 cells which appeared as dark blue, stained areas with the lack of any clear spots. These results were similar to those reported by Frey[3].

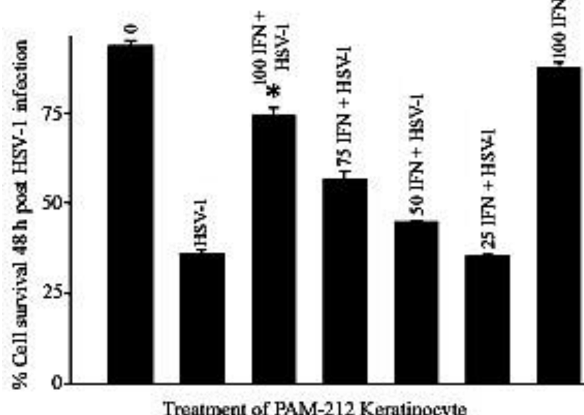
MOI	VERO CELL	PAM-212	HEL-30
0.1			
0.01			
Control			

(Figure 2) cells response to 0.1 and 0.01 MOI of HSV-1

Vero cells, HEL-30, and PAM-212 were infected with different MOI of HSV-1. Cells were stained with crystal violet in 70% methanol. Arrows denote to the viral plaques. The effect of IFN-γ on HEL-30 and PAM-212 keratinocytes (figure 2).

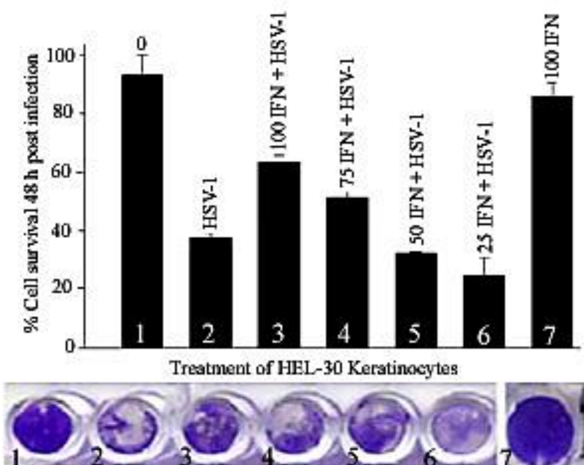
As known that IFN- γ has an antiviral effect, we attempted to protect HEL-30 & PAM-212 keratinocytes by treating them with different concentrations of IFN-

γ. These cells showed different response to IFN-γ treatment. The antiviral effect of IFN- γ was greater in PAM-212 cells which showed about 40 folds increase in the cells viability with the use of 100 unit, 25 folds with 75 units, and 10 folds with 50 units (figure 3).



(Figure 3) Effect of IFN-γ on HSV-1-infected PAM-212 keratinocytes

However, HEL-30 cells did not show much difference in the cell survival even with the use of high concentrations of IFN- γ (20 folds with 100 and 10 folds with 75 units) (figure 4). Moreover, higher concentration of IFN-γ provided more protection as seen by increased cell density.



(Figure 4) Effect of IFN-γ on HSV-1-infected HEL-30 keratinocytes

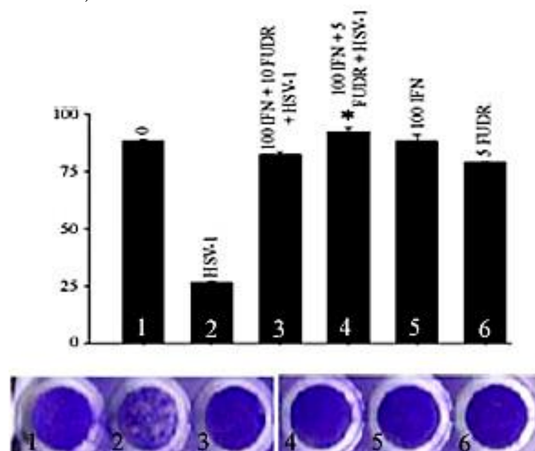
Cells were treated with various concentrations of IFN-γ for 24h then infected with 0.01MOI HSV-1 for 2h. 24h after infection. The cells were stained and image J was used to count the cells viability. Error bars indicate standard error of the means. There were about a 40 fold differences between cells treated with100 IFN-γ unit (*) and untreated, uninfected cells (0) ($p < 0.001$). Note that the 0 bar denotes the viability in culture of untreated, uninfected cells.

HEL-30 were treated with various concentrations of IFN-γ for 24h then infected with 0.1 MOI of HSV-1 for 2h. 24h after infection, the cells were stained and the images analyzed using the Image J program to determine cell density (viability). Error bars indicate standard error of the means. There were no significant differences between the viability of IFN-γ treated cells and untreated, infected cells (0). The cell viability after treatment with 100ug/ml IFN-γ increased 25-fold relative to virus infected cells ($p < 0.001$). None of the differences among the HSV-1 infected cells was statistically significant. Note that the 0 bar denotes the viability in culture of untreated, uninfected cells. These results mirror those observed by Frey et al., for the PAM-212 keratinocytes after infection with 0.1 MOI of HSV-1. A difference from the Frey study was seen for the HEL-30 cells in which approximately 20% of the keratinocytes survived HSV-1 infection with 0.1 MOI of virus. In Frey's study, none of the keratinocytes survived an MOI of 0.1. This difference may reflect differences in the source of HEL-30 cells.

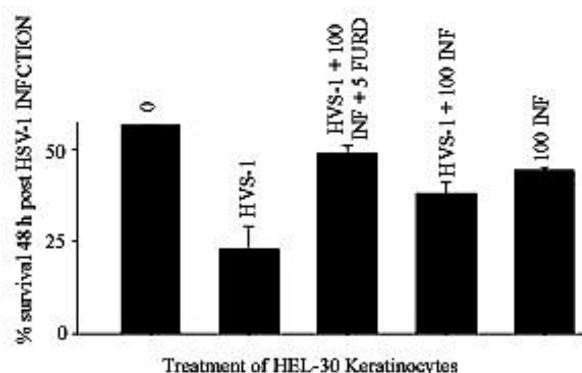
The HEL-30 cells in this study were derived from a stock received from WPAFB, while Frey's were supplied by National Institutes of Health. The two mitotic inhibitors induce differences in the keratinocyte cell lines in a time and concentration-dependent manner. The effect of FUDR on HEL-30 and PAM-212 Keratinocytes. Since FUDR acts as a mitotic inhibitor, the expectation was that the HSV-1 infection would not cause a cell death for the FUDR-treated cells. FUDR treatment would promote a cell proliferation. To determine the effect of FUDR on keratinocytes, cells were treated with 100 units of IFN-γ for 24h, followed by exposure to either 10 or 5µg/ml FUDR before viral infection. The 5 µg/ml concentration permitted about 65 fold increase in PAM-212 cell viability comparing to untreated cells (figure 5) with less activity on HEL-30, (figure 6). These variations in the response could due to the fact that different keratinocytes differ in their susceptibility to HSV-1 infection.

PAM-212 keratinocytes were plated in 96 well plate, and treated when they were about 80% confluence with 100 u/ml of INF-γ for 24h followed by 5 or 10µg/ml of FUDR treatment for an hour before infection with the 0.01 MOI of HSV-1.

There were statistically significant differences between 100 u/ml IFN-γ and 5 µg/ml FUDR treated cells (*) and untreated, uninfected cells (0) ($p < 0.001$). Note that the 0 bar denotes the viability in culture of untreated, uninfected cells.



(Figure 5) The effect of FUDR on PAM-212 keratinocyte



(Figure 6) The effect of FUDR on HEL-30 keratinocyte

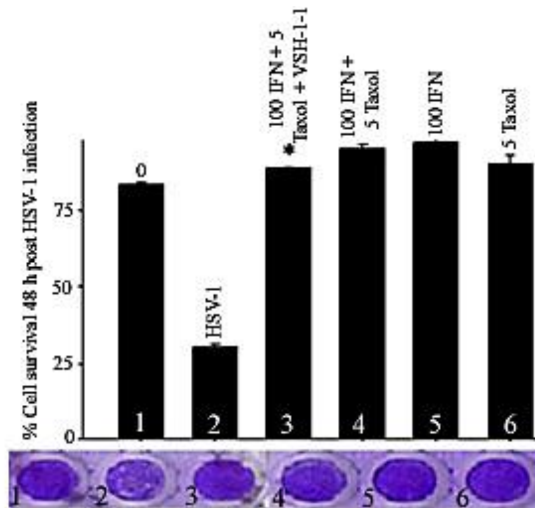
HEL-30 keratinocytes were plated in 96 well plates, and treated with 100µg/ml of IFN-γ when the cell confluence was about 80% for 24 hours. Then 5 µg/ml of FUDR was added for an hour, followed by infection with the 0.1 MOI. There was a 25 fold difference between IFN-γ and FUDR treated cells (*) and untreated, uninfected cells (0) ($p = 0.013$). Note that the 0 bar denotes the viability in culture of untreated, uninfected cells. As shown in (figure 7), PAM-212 treated with FUDR maintains its cell morphology and the percentage of growth. However, HEL-30 treated cells showed some decrease in the rate of proliferation compared to untreated cells. Moreover, HEL-30 cells formed more regular polygons.

	PAM-212	HEL-30
Cell Only		
Cell + HVS-1 at 2,0 MOI		
Cell + FUDR		
Cells treated with FUDR and infected with 2,0 MOI HVS-1 (12h post infection)		
Cells treated with FUDR and infected with 2,0 MOI HVS-1 (16h post infection)		

(Figure 7) The effect of FUDR on PAM-212 and HEL-30 morphology and proliferation

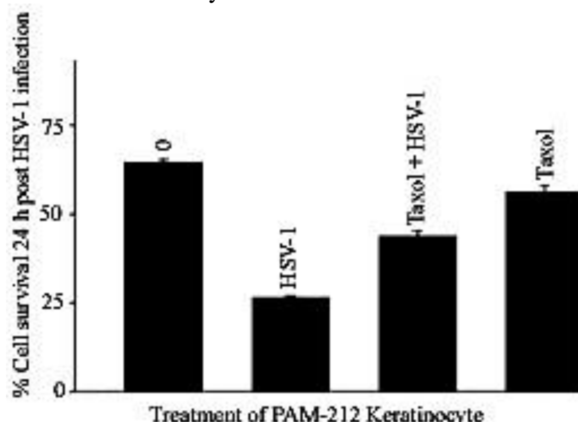
These pictures were taken with a 10X objective lens. The effect of paclitaxel on PAM-212 and HEL-30.

To examine the effect of paclitaxel on PAM-212 keratinocytes, cells were treated with IFN-γ and paclitaxel before HSV-1 infection. PAM-212 treated cells showed 50 folds increase in the cell growth compared with untreated cells (figure 8).



(Figure 8) the effect of Taxol on PAM-212 keratinocytes

To see if this effect was related to the paclitaxel or IFN-γ, the cells were treated with paclitaxel only. As shown in (figure 9), paclitaxel induced cell proliferation (20 folds increase) even in the absence of IFN-γ. These results indicate that paclitaxel can inhibit the cell death caused by HSV-1.

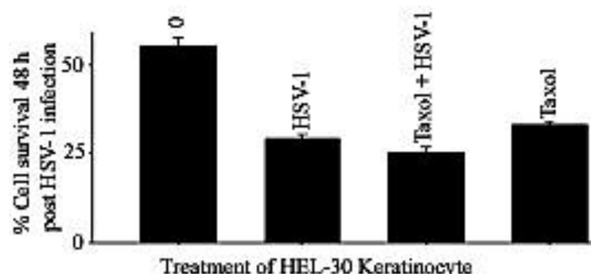


(Figure 9) The effect of Taxol on PAM-212 keratinocytes without the IFN- γ treatment

PAM-212 keratinocytes were plated in 96 well plate, and treated when they are about 80% confluence with 100u/ml of IFN-γ for 24h followed by treatment with 5 µg/ml taxol for an hour before infection with the virus at 0.1 MOI. There were statistically significant differences between IFN-γ and FUDR treated cells (*) and untreated, uninfected cells (0) ($p < 0.001$). Note that the 0 bar denotes the viability in culture of untreated, uninfected cells.

PAM-212 cells were treated with 5µg/ml of Taxol for 30 min before HSV-1 infection. Taxol- treatment provided a 20 fold increase (*), even in the absence of IFN-γ ($p = <0.001$). Note that the 0 bar denotes the viability in culture of untreated, uninfected cells. Because IFN-γ potentiated the lytic effect in HEL-30 cells at an MOI of 0.1 as showed in (figure 3), this cell line was not examined further following treatments with paclitaxel and IFN-γ. As shown in (figure 10), paclitaxel had a toxic effect on HEL-30 which ap-

peared by the decrease of cell survival by 20 folds even in the absence of HSV-1.



(Figure 10) The effect of paclitaxel/taxol on HEL-30 keratinocyte

HEL-30 keratinocytes were plated in 96 well plates. When they were about 80% confluent, the cells were treated with 5µg/ml of taxol for 30 min, followed by infection with the 0.1 MOI of HSV-1. Taxol treatment had a toxic effect on HEL-30 cells (*) compared to untreated, uninfected cells (p = <0.001). Note that the 0 bar denotes the viability in cultures of untreated, uninfected cells.

In cultures of paclitaxel-treated keratinocytes, changes in the proliferation rate and morphology were observed (figure 11).

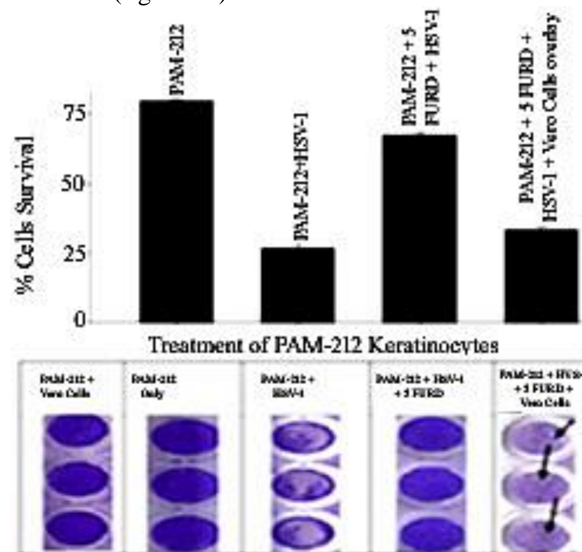
	PAM-212	HEL-30
Cell Only		
Cells + at an MOI of 2.0 HSV-1		
Cells + Taxol		
Cell treated with Taxol and infected with 2.0 MOI HSV-1 (12h post infection)		
Cell treated with Taxol and infected with HSV-1 (16h post HSV-1 infection)		

(Figure 11) The effect of paclitaxel/taxol on PAM-212 and HEL-30 morphology and proliferation

These changes which were different depending on the cell line were anticipated because paclitaxel causes microtubule's alteration. HEL-30 cells were less susceptible to the anti-mitotic effect of paclitaxel than were PAM-212 cells. This may be due to the reaction level between the cells and the paclitaxel or any other treatments depending on the tumor type which is erythroleukemia in HEL-30 and cutaneous squamous cells carcinoma in PAM-212 cell line. It also could due to the variations in the degree of cell–cell contact formation, depending on cell density in the culture dish[6].

HEL-30 keratinocyte exhibited some changes in their morphology; these cells were more rounded than untreated, uninfected cells and lost their plastic adhesion. These observations were made microscopically 24h after treatment, as well as 12h and 16h after HSV-1 infection with an MOI of either 0.1.or 2.0 in which about 25%, 60% and 80% respectively of cells were dead. Nevertheless, PAM-212 keratinocytes were much less affected by the apoptotic effect and showed that they were more likely to go toward cell cycle arrest (figure 12). These results were comparable to results reported by Kim and his colleagues' using different cell lines[7].

These pictures were taken with a 10X objective lens Attempt to rescue virus from FUDR-treated PAM-212 cells[8]. Overlayed single cell suspensions of trigeminal neuronal cells with fibroblasts to rescue latent HSV-1 virions from the trigeminal neuronal cells. Based on this observation, susceptible Vero cells were used to overlay the PAM-212 cell cultures infected with HSV-1 in attempt to release the virus from PAM-212 cells (figure 12).



(Figure 12) Effect of HSV-1 infection on FUDR-treated PAM-212 keratinocytes

If the virus established latency inside HSV-1- infected, FUDR- treated PAM-212 cells, we will have a decrease in the cell survival as a result of Vero cells overlay. Note that viral plaque formation is apparent (marked with arrows) in the Vero cells overlayed onto HSV-1- infected, FUDR- treated PAM-212 cells. An alternate explanation for this observation may be that the virus exiting from the PAM-212 cells to infect the Vero cells causes trauma to the PAM-212 cells. The staining of this set of wells was lighter and the Vero cells overlay induced 40 fold decrease in the cell survival compared with the un-overlayed cells. These results suggest that the infected Vero cells may be releasing enzymes which damages the PAM-212 cells.

Further experimentation is needed to confirm that this viral infection reflects latency rather than surface contamination with live virus. Polymerase chain reaction has to be done looking for LAT, which is the main sign of latency.
Fewer cells survived after Vero cells overlay (*) compared to FUDR treated- HSV-1 infected cells (p= <0.001). Note that the 0 bar denotes the viability in culture of untreated, uninfected cells.

CONCLUSION AND RECOMINDATION

Primary infection, latent infection, and reactivation are the main stages that characterized HSV-1. However, the stages leading to HSV-1 latency is still unclear. If it is possible to develop a latent stage in keratinocytes, then it may be possible to dissect these events to understand progression from initial infection to latency as seen in neuronal cells. Since most of the antiviral drugs target the lytic state, targeting the virus in the latent state is considered one of the new approaches for potential treatments. In this study, two mitotic inhibitors were used to render the keratinocytes susceptible to a latent HSV-1 infection.
In this experiment , FUDR treatment of both keratinocyte cell lines permitted an increased survival of HSV-1- infected keratinocytes, especially for PAM-212 keratinocytes. This cell line was further examined for the presence of sequestered virus after treatment with FUDR and infection with HSV-1. Knickelbein et al. were abled to rescue HSV-1 from latency in murine trigeminal ganglia by co-culture with susceptible fibroblasts. As shown in (figure 12), viral plaques were seen in PAM-212 cells 48h after infection with HSV-1 and 24h after overlay with susceptible Vero cells suggesting that this approach warrants further study. This study sets the stage for future work in the laboratory confirming the usefulness of the latency model[(8)].
Further examination of the effect of FUDR on keratinocytes using different times of exposure (2h, 4h, 6h, and 24h) is needed. Since Rich and his colleagues found that the inhibitory effect of FUDR is reversible[(9)], HSV-1 infection of keratinocytes should be examined at different times after FUDR treatment.
Paclitaxel was also used as a mitotic inhibitor in these keratinocyte cells line in attempts to induce viral latency. The concentration of paclitaxel used (5μg/ml) was toxic to both cell lines. Since Choritz et al., have found that the growth and migration inhibition resulted from the exposure to paclitaxel/taxol are a dose and time dependent. Low concentrations of paclitaxel induce less cell death, further investigation with lower concentrations of paclitaxel (1μg/ml, 0.5 μg/ml, 0.01 μg/ml or lower) at different time of exposure is needed to see if these low concentrations could induce a mitotic inhibition instead of cell death[(10)].
In the latency process, silencing the lytic gene expression and blocking the host cellular and humoral immune response have to be considered to achieve this goal. If treatments of keratinocyte cell lines with these mitotic inhibitors render the cells susceptible to herpes virus latency, expression of latency-associated transcript (LAT) must be demonstrated in these cells. Future studies should include determining the polymerase chain reaction products for early viral genes (ICP4 and ICP0) as well as latent-associated transcript (LAT) in the keratinocyte when they are in the post mitotic state. Initial attempts to demonstrate LAT in PAM-212 following treatment with FUDR and HSV-1 infection were performed at 12h and 16h post infection. Since ICP0 and ICP4 genes (immediate early, IE) are expressed early in infection time points of 5h and 16h post infection should be examined. ICP0 and ICP4 expression should predominate in the 5h samples and LAT, if present, should predominate in the 16h sample to demonstrate latency. Future studies should include determining the polymerase chain reaction products for early viral genes (ICP4 and ICP0) as well as latent-associated transcript (LAT) in the keratinocyte when they are in the post mitotic state.

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