INVESTIGATION OF HERPES SIMPLEX-1 LATENCY IN VIVO AND IN VITRO

Summary of PhD thesis

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Publications directly related to the subject of the dissertation

I.   Valyi-Nagy K, **Dósa S**, Kovacs SK, Bacsa S, Voros A, Shukla D, Folberg R, Valyi-Nagy T. Identification of virus resistant tumor cell subpopulations in three-dimensional uveal melanoma cultures. Cancer Gene Ther., 17(4):223-234 (2010)

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II.   **Dósa S**, Castellanos K, Bacsa S, Gagyi E, Kovacs SK, Valyi-Nagy K, Shukla D, Dermody TS, Valyi-Nagy T. Chronic progressive deficits in neuron size, density and number in the trigeminal ganglia of mice latently infected with herpes simplex virus. Brain Pathol., 21(5):583-593 (2011)

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1. Introduction

Amongst human diseases caused by pathogen microorganisms, viral pathogens play a significant part. An acute infection generally means that the infected individual shows symptoms and after that the infective virus is eliminated completely by the host´s immune system. A chronic or persistent infection in general can be defined as an infection that is present in a given individual for a long time.

On the cellular level a viral infection can be abortive when some viral components are synthesized but no infective virus is assembled and/or released. An infection is defined productive when new infectious viruses are produced. This process results generally in the death of the host cell called lytic infection. However, there are some viral families and species which have the ability to maintain a different route in the lifecycle. In this case there is no immediate lysis of the host cells. The viral genome can exist in the host cell for a longer period of time. The characteristic hallmark of this type of viral cycle is the reversibility where under certain circumstances the expression of the viral genome can be activated resulting in the productive lytic cycle. This different way in the life cycle called latent (dormant) life cycle or infection.

The clinical and epidemiological significance of viral latency is highly important since this type of infection serves as potential reservoir from which dissemination of the pathogen to a new host can occur. On the other hand, such infections could play a role in the

development of some human malignant and benign tumors and thirdly, because the reactivation of the latent virus might cause acute or chronic progressive disorders in the original host. The possible consequences of latency show divergent patterns. The most common form of the outcome is the reactivation following different activator signals (i.e. stress, sunlight) to cause an acute infection and production of viral progeny. A less frequent but more severe consequence is the transformation of the latently infected cell which results in a virus induced formation of malignant or benign tumors. The transformation can be caused by viral gene products which may exert their effect by interfering with particular cellular proteins or by insertional mutagenesis.

One of the most extensively studied virus families known to maintain latency is *Herpesviridae*. The HSV-1 – which belongs to the family of *Herpesviridae* and was the subject of our investigations – is a neurotropic virus that establishes latency in sensory neurons innervating the site of primary infection and has the capacity to sustain a life-long latent infection and it can also be reactivated from those neurons in a periodical manner. The goal of our work was to investigate the effect of the HSV-1 latent infection using two model systems.

First, we examined *in vivo* the role of the HSV-1 latent infection in mouse TG-s. Studies have shown chronic behavioral and neurological deficits and chronic pain in these animals but chronic progressive quantitative alterations remained poorly understood. Using morphometric studies we tested whether HSV-1 latent infection has an effect on those above mentioned quantitative indicators in healthy adult mice.

Our second aim was to explore *in vitro* virus resistance/latency of distinct tumor cell subpopulations in 3D tumor cell cultures and the mechanisms of ECM-mediated virus resistance of tumor cells. Previous studies have indicated that tumor cells grown in 3D cultures are also more resistant to viral, including HSV-1 and adenovirus-mediated oncolytic therapy, than cells grown in 2D culture.

2. Aims

2.1 To investigate the HSV-1 latency and the consequent quantitative alterations on morphologic level using *in vivo* model

- To examine the effect of latent HSV-1 infection on mean neuron diameter, neuronal nucleus diameter, neuron density and neuron number in trigeminal ganglia derived from adult BALB/c mice in association with neuronal injury.

2.2 To explore HSV-1 latency in tumor cells and the effect of extracellular matrix concerning latently infected tumor cells

- To explore whether 3D tumor cell cultures can be used to identify morphologically distinct tumor cell populations that have increased resistance to HSV-1.

- To examine whether 3D tumor cell cultures can be used to learn about mechanisms of ECM mediated tumor cell resistance to HSV-1 oncolytic therapy.

3. Material and methods

3.1 Investigation of HSV-1 latency and the consequent quantitative alterations on morphologic level using *in vivo* model

3.1.1 Inoculation of mice

BALB/c mice were inoculated with 1×10^5 P.F.U. of HSV-1 17+ or sterile tissue culture medium per eye after superficial corneal scarification. Groups of HSV-1-infected or mock-infected mice were euthanized at 1, 12 and 31 weeks after inoculation and TG were aseptically removed, fixed and embedded in paraffin.

3.1.2 Immunohistochemical detection of HSV-1 proteins

Multiple 6-μm-thick tissue sections of the paraffin-embedded TG tissues were deparaffinized then endogenous peroxidase activity was quenched. HSV-1 antigens were detected using a 1:1000 dilution of a polyclonal anti-HSV-1 antiserum raised in a rabbit. Tissue sections were incubated with primary antibody before the addition of biotinylated anti-rabbit immunoglobulin secondary antibody and HRP labeled avidin and H_2O_2 and DAB as substrate.

3.1.3 *In situ* hybridization for HSV-1-LAT gene expression

In situ hybridization was performed using a nick-translated ^{35}S-labeled DNA probe specific for HSV-1 latency-associated transcript (LAT) (Bst2±Bst2 fragment) and 6-μm-thick tissue sections. After 2 to 4 days of exposure, slides were developed with D19 and stained with hematoxylin and eosin. The specific activities of the nicktranslated ^{35}S-labeled probes were 1×10^8 to 2×10^8 cpm/μg.

3.1.4 Determination of mean neuron diameter, neuron nucleus diameter, neuron density, TG volume, and neuron number per TG

For the determination of mean neuron diameter and mean neuron nucleus diameter, multiple 6-μm-thick tissue sections were scanned at 200 x magnification using the ImageScope® System and analyzed with the aid of Aperio image analysis software. The largest diameter and the largest nuclear diameter of 100 randomly chosen neurons were measured manually by an observer blinded to the nature and time of inoculation. Measurements were made for 7 to 12 TG sections derived from five mice yielding 700 to 1200 measurements per group.

For neuronal cell density measurements, multiple 20-μm-thick sections were cut, stained with hematoxylin and viewed using a 60x objective under an Olympus BH-2 microscope. Five TG (randomly selected left or right) from five mice were examined. Neuronal cell density determinations were made with the aid of image analysis software, digital video camera and a computer system according to a published stereological optical dissector method. Neuronal density was determined with using Tandrup's review concerning dorsal root ganglion as a guiding principle. The total value of n_v (neurons per volume) for a

TG was then determined from: $n_v = \dfrac{\Sigma?}{?\cdot\Sigma?????\ ???}$ where ΣQ^- is the sum of the dissector neurons in the total dissector volumes, $a_{(frame)}$ is the area of the counting frame, h is the height of the optical dissector and p is the number of points per dissector.

The volume of TG was calculated using image analysis software and digital video camera to draw manually the contour of each analyzed TG section at a magnification of 5x and to measure automatically its area in mm^2. The total volume of ganglia was calculated by multiplying the surface area by the thickness of the section and by the number of sections.

The number of neurons per TG was calculated based on neuron density and TG volume as follows: $N = n_v \ x \ V$, where N corresponds to neuron number per TG, n_v stands for neuron density and V for TG volume.

3.1.5 Statistical analyses

Statistical analyses were performed with the STATISTICA software (version 8.0) for Windows. Normality was tested using the Kolmogorov–Smirnov test. All variables were distributed normally. Homogeneity of variance was determined using F-test and Levene's test was considered violated when this test yielded $p < 0.05$. All variances were homogeneous. Data were assessed using independent samples T-test and repeated measures analysis of variance (ANOVA) followed by Scheffe's post-hoc test. Differences between the means were considered statistically significant if $p < 0.05$. The results are expressed as means ± standard deviation (SD) values.

3.2 Investigation of HSV-1 latency in tumor cells and the effect of extracellular matrix concerning latently infected tumor cells

3.2.1 Determination of susceptibility of uveal melanoma cells to wt HSV-1 (KOS) and HSV-1 K26GFP-mediated destruction in 2D and 3D cultures

C918 and OCM1 uveal melanoma cells were grown in the presence (three-dimensional; 3D cultures) or absence of Matrigel (two-dimensional; 2D cultures). When 2D cultures of C918 and OCM1 cells reached approximately 70% confluency Matrigel was layered on the cells. Matrigel formed an approximately 1 mm thick layer matrix on the cells (3D cultures). Next, both 2D and 3D cultures were infected with wt HSV-1(KOS) (M.O.I. of 0.5) or HSV-1 K26GFP (M.O.I. of 0.5) or were mock infected with PBS and were observed

daily for a 4-week period under an inverted microscope for evidence of viral cytopathic effects and green fluorescent protein (GFP) expression. The day when at least 95% of the melanoma cells were destroyed was noted.

3.2.2 Determination of HSV-1 spread through Matrigel

3D cultures of C918 and OCM1 uveal melanoma cells were either inoculated with HSV-1 K26GFP (M.O.I. of 0.5) or were mock infected with PBS. Cultures were then further incubated for up to 2 weeks and were observed regularly under an inverted fluorescence microscope for evidence of GFP expression.

3.2.3 Placement and culturing of earlier HSV-1 inoculated uveal melanoma cells in 2D or 3D environments

2D cultures of C918 and OCM1 uveal melanoma cells at 70% confluence were exposed to sterile PBS (mock infection) or HSV-1 K26GFP (M.O.I. of 0.5). After inoculation cells were scraped and cell pellets were resuspended in culture medium. For 3D cultures, an approximately 0.2 mm thick Matrigel matrix layer was formed on the cells. Cell suspensions of HSV-1 K26GFP or mock-infected uveal melanoma cells were mixed with Matrigel 1:1 and poured on the Matrigel-coated wells. 2D and 3D cultures were then observed regularly under an inverted fluorescence microscope for up to 4 weeks. Evidence of GFP expression and the percentage of GFP-expressing cells were determined by counting the number of GFP-positive and GFP-negative cells in 16 high power microscopic fields.

4. Results

4.1 Investigation of HSV-1 latency and the consequent quantitative alterations at morphologic level using *in vivo* model

4.1.1 Characterization of TG tissues derived from mice at 1, 12 and 31 weeks following corneal HSV-1 inoculation

To obtain TG tissues at various stages of HSV-1 infection, mice were inoculated on the cornea with either HSV-1 or sterile tissue culture medium. Animals surviving beyond 3 weeks of virus inoculation demonstrated no signs of encephalitis and developed normally. Groups of five randomly chosen HSV-1-infected or mock-infected mice were euthanized at 1, 12 and 31 weeks after inoculation.

No HSV-1 protein expression was detected in the TG of mock-infected mice at any time point. 1 week after HSV-1 inoculation, HSV-1 proteins were detected in all 10 TG by immunostaining, consistent with replication of HSV-1. In TG derived from animals euthanized at 12 and 31 weeks after corneal HSV-1 inoculation, HSV-1 protein expression was not detected.

Abundant LAT RNA expression was detected by in situ hybridization in TG tissues of HSV-1-infected mice euthanized at 12 and 31 weeks after virus inoculation.

Sections of TG stained with hematoxylin and eosin at 1, 12, and 31 weeks after HSV-1 inoculation revealed chronic inflammatory changes. Inflammation was not detected in the TG of mock-infected animals. Both acute and latent HSV-1 infection in the murine TG was associated with inflammation.

4.1.2 Mean neuron diameter, mean neuronal nucleus diameter, and the number of neurons increase in the TG of mock-infected BALB/c mice

Mean neuron diameter and mean neuronal nucleus diameter gradually increased in the TG of control mice after mock infection. Mean neuronal nucleus diameters at 6, 17, and 36 weeks of age were 9.15 ± 2.2 μm (n=700), 9.27 ± 1.8 μm (n=700), and 10.33 ± 2.0 μm (n=900), respectively. Increases in mean neuron diameter and neuronal nucleus diameter did not differ statistically between 6 and 17 weeks (p=0.3782 and 0.2794, respectively), but were significantly different at 6 and 36 weeks and 17 and 36 weeks. These findings are consistent with growth of TG neurons during the observation interval.

Neuron density in the TG decreased while TG volume increased in mock-infected mice during the period of observation. The combination of these changes resulted in a gradual increase in the mean number of neurons per TG between 6 and 36 weeks of age. Thus, TG volume and neuron number per TG increase, while neuron density decreases in the TG of mice between 6 and 36 weeks of age.

4.1.3 Productive HSV-1 infection in the TG of mice is associated with increased mean neuron diameter and neuronal nucleus diameter and decreased neuron density

In the TG of mice euthanized 1 week after corneal HSV-1 inoculation, mean neuron diameter and mean neuronal nucleus diameter were greater than that detected in TG derived from mice 1 week after mock infection. Analysis of TG sections derived from mice 1 week following virus inoculation and immunostained for HSV-1 proteins indicated that mean neuron diameter and neuronal nucleus diameter were significantly greater in HSV-1 protein expressing neurons than in HSV-1 antigen–negative neurons.

Mean neuron density was 8.69% less in TG derived from HSV-1-inoculated mice than in mock-infected controls. Mean TG volume was similar in HSV-1- and mock-infected mice. The mean number of neurons per TG was 17 000 ± 1400 in virus-infected vs. 18 600 ± 1000 in mock-infected TG, an 8.61% decrease. This decreasing tendency in neuron number per TG did not reach statistical significance. Collectively, these findings are consistent with neuron destruction by productive HSV-1 infection in the TG.

4.1.4 Latent HSV-1 infection in the TG of mice is associated with decreased mean neuron diameter, neuronal nucleus diameter and neuron density and number

In the TG of mice euthanized 12 weeks after corneal HSV-1 inoculation, mean neuron diameter, mean neuronal nucleus diameter, neuron density and mean neuron number per TG were all significantly less than those in age-matched mock-infected control mice. Specifically, mean neuron diameter was 22.25 ± 4.6 µm vs. 22.98 ± 4.9 µm detected in age-matched mock-infected controls, a 3.17% decrease. Mean neuronal nucleus diameter was 9.07 ± 1.8 µm vs. 9.27 ± 1.8 µm in controls, a 2.15% reduction. Neuron density was 8.94% less. Mean TG volume did not differ significantly. However, the mean number of neurons per TG was significantly less in HSV-1-infected mice than in mock-infected mice, a 12.25% decrease. Thus, neuron size and number are decreased in the TG of mice 12 weeks after HSV-1 corneal inoculation.

In the TG of mice euthanized 31 weeks after corneal HSV-1 inoculation, mean neuron diameter, mean neuronal nucleus diameter, neuron density, and mean neuron number per TG were all significantly less than those detected in age-matched mock-infected control mice. Specifically, mean neuron diameter was 23.55 ± 5.4 µm vs. 25.16 ± 5.0 µm in age-matched mock-infected controls, a 6.4% decrease. Mean neuronal nucleus diameter was 9.76 ± 2.0 µm

vs. 10.33 ± 2.0 μm in controls, a 5.5% reduction. Neuron density was 13.3% less in comparison with those from mock-infected mice. Mean TG volume did not differ significantly. However, like the TG from mice at 12 weeks after HSV-1 inoculation, the number of neurons per TG was significantly less in HSV-1-infected mice than in mock-infected. Specifically, the total number of neurons was 33.620 ± 1600 in virus-infected vs. 40950 ± 3100 in mock-infected TG, a 17.9% relative decrease.

Deficits in mean neuron diameter, neuron nucleus diameter, neuron density, and neuron number per TG relative to age-matched mock-infected controls were all more substantial at 31 weeks than at 12 weeks after corneal virus inoculation. As only latent HSV-1 infection was detected in TG neurons at 12 and 31 weeks after corneal virus inoculation, the progressive deficits in neuron size, density, and number between these times are consistent with chronic progressive neural injury during latent HSV-1 infection.

4.2 Investigation of HSV-1 latency in tumor cells and the effect of extracellular matrix concerning latently infected tumor cells

4.2.1 Rapid destruction of 2D and a delayed and incomplete destruction of 3D uveal melanoma cultures by wt HSV-1 and HSV-1 K26GFP

To examine the process of HSV-1 infection in 2D and 3D cultures of OCM1 and C918 cells, the cells were inoculated with wt HSV-1 strain KOS or HSV-1 strain K26GFP. Cultures were followed for evidence of cytopathic effects and virus replication for 4 weeks by an inverted fluorescence microscope. 2D cultures were completely destroyed by both HSV-1 strains within a few days with no evidence of surviving cells. In K26GFP inoculated 2D cultures, 20–50% of tumor cells showed GFP expression by 1 day post infection (p.i.), and after 4-5 days p.i., complete viral replication-mediated destruction of these cultures was observed. In contrast, virus inoculated 3D cultures showed delayed and incomplete destruction. Importantly, a portion of both OCM1 and C918 cells seemed to survive HSV-1 KOS and HSV-1 K26GFP infection in 3D cultures for up to 4 weeks. 3D cultures of both OCM1 and C918 showed a significant number of GFP-negative cells throughout the 4-week observation period.

4.2.2 Matrigel impairs HSV-1 spread

To determine whether inhibition of HSV-1 spread through Matrigel was a mechanism responsible for the absence of virus replication (GFP expression) in some tumor cells in 3D cultures, monolayers of OCM1 and C918 cells were covered with a thick layer of Matrigel and then HSV-1 K26GFP was placed on the Matrigel surface. As controls, monolayers of OCM1 and C918 cells without a Matrigel cover were also inoculated with the same amount of virus. Virus inoculated uveal monolayers with no Matrigel cover showed widespread GFP expression by 18–24 h p.i. and were completely destroyed within a few days. In contrast, cells covered with Matrigel and inoculated with HSV-1 K26GFP showed continued viability and growth during a 2-week observation period with no evidence of GFP expression. These findings indicate that Matrigel inhibits HSV-1 spread.

4.2.3 Extracellular matrix (ECM) mediates inhibition of HSV-1 replication after virus entry into tumor cells

To determine whether inhibition of viral replication after virus entry into tumor cells is a mechanism responsible for the absence of virus replication in some tumor cells in 3D cultures, uveal melanoma cells were first inoculated with HSV-1 K26GFP under 2D conditions and were then cultured under either 2D or 3D conditions. After the plating of earlier mock-infected OCM1 and C918 cells under either 2D or 3D conditions, tumor cells showed normal growth and no evidence of GFP expression was observed. After the plating of earlier HSV-1 K26GFP inoculated OCM1 and C918 cells on tissue culture dishes, tumor cells established monolayers (2D cultures) that were completely destroyed by virus replication within a few days. At 18 h p.i., $29.53 \pm 15.01\%$ of OCM1 cells and $40.33 \pm 15.17\%$ of C918 cells showed fluorescence (evidence of HSV-1 replication). Nearly all OCM1 cells were GFP positive by 72 h and nearly all C918 cells were GFP positive by 96 h. These findings are consistent with replication of input virus in infected cells at 18 h p.i. and virus spread to all cells in the following days. Culturing of earlier HSV-1 K26GFP inoculated OCM1 and C918 cells in 3D was initially associated with single cells suspended in matrix. More than half of the cells showed morphologic changes during the first day of the establishment of the 3D cultures including rounding and a minority of cells showed morphological features of apoptosis. At 18 h p.i., the numbers of OCM1 and C918 cells in the virus-infected 3D cultures were 12.5 and 8.5% lower than those in their mock-infected 3D counterparts. At 18 h p.i.,

2.80 ± 2.75% of OCM1 cells and 2.22 ± 3.05% of C918 cells showed fluorescence in the HSV-1-infected 3D cultures. The number of GFP-expressing cells did not increase the following days. Thus, at 18 h p.i., a significantly smaller percentage of cells showed evidence of virus replication in 3D cultures than what was expected on the basis of the amount of input virus. These observations indicate that the ECM can mediate the inhibition of HSV-1 replication after virus entry into tumor cells.

5. DISCUSSION

A general biological characteristic of herpes viruses is their ability to sustain lifelong infection of the host despite an operable immune response. The complex mechanism of the occasional re-activation and shedding of the virus then allows the infection of new hosts and maintains high levels of viral infection within the population.

In our first model system we used quantitative morphometric analyses to test whether latent HSV-1 infection affects mean neuron diameter, neuronal nucleus diameter, neuron density, and neuron number in the TG of mice.

We show here using careful morphometric analyses that in healthy adult mice, mean neuron diameter, mean neuron nucleus diameter, TG volume, and mean number of neurons per TG gradually increase between 6 and 37 weeks of age. The detection of increasing neuron diameter, neuronal nucleus diameter, and TG volume is not surprising and are similar to those made in several reports of gradually increasing numbers of mature neurons in the sensory ganglia of healthy adult rats.

Numerous previous studies indicate that corneal inoculation of immunocompetent mice with HSV-1 is followed by virus replication in the TG for about two weeks, which in turn is followed by the establishment of life-long HSV-1 latency in the TG. Consistent with these reports, we detected productive HSV-1 infection in the TG at 1 week after virus inoculation and evidence of latent HSV-1 infection in the TG at 12 and 31 weeks after virus inoculation. In the TG of mice euthanized 1 week after corneal HSV-1 inoculation, mean neuron diameter and mean neuronal nucleus diameter increased, while neuron density and mean neuron number per TG decreased relative to age-matched mock-infected controls. These findings are not surprising, neuronal death in the TG of mice during the first few weeks of infection is well documented, which would account for the decrease in neuron number.

The key finding of our study is that in the TG of mice 12 and 31 weeks after HSV-1 inoculation (at 17 and 36 weeks of age), mean neuron diameter, neuronal nucleus diameter, neuron density, and mean neuron number per TG were significantly less than those in age matched mock-infected control TG. Interestingly, the deficits in neuron diameter, neuronal nucleus diameter, neuron density, and neuron number in latently infected TG were more substantial at 36 weeks than at 17 weeks of age. Mean neuron diameter, mean neuronal nucleus diameter, and mean neuron number per TG increased in absolute numbers between 17 and 36 weeks in latently infected mice. These increases were substantially less than those in mock-infected controls and these relative deficits all increased during latency.

These findings indicate that latent HSV-1 infection is associated with chronic progressive deficits in neuron size, density, and number in the nervous system of an immunocompetent host.

Mechanisms by which latent HSV-1 infection leads to chronic progressive alterations in neuron size, density, and number in the nervous system are not clear from our study. As spontaneous HSV-1 reactivation occurs in latently infected mice, albeit rarely, it is possible that reactivation events contribute to changes in neuronal size and number. Persistent inflammation during HSV-1 latency in the murine TG may cause neuronal injury, possibly mediated by cytokines and oxidative stress. Indeed, the extent of neuronal injury during latent infection in our current study seemed to correlate with the extent of inflammation in the TG. These viral and inflammatory processes may injure mature neurons or interfere with ongoing neurogenesis and neuronal maturation in the TG.

While mice surviving the acute phase of experimental HSV infection do not show signs and symptoms of encephalitis, numerous studies documented chronic behavioral and neurological deficits and chronic pain in these animals. In the current study, mice were observed daily for signs and symptoms of encephalitis, but detailed neurological and behavioral studies were not performed. These studies may lead to major new insights into mechanisms by which latent viral infections contribute to the pathogenesis of chronic neurological and neuropsychiatric diseases and foster development of virus-specific intervention strategies.

In our second model system we used 2D and 3D cultures of uveal melanoma cells to identify morphologically distinct tumor cell populations with increased resistance to HSV-1. We show that tumor cells forming vasculogenic mimicry patterns and multicellular spheroids and cells that invade the ECM individually have increased resistance to HSV-1. Furthermore, we show that mechanisms of tumor resistance against HSV-1 in the 3D environment include

impaired virus spread in the ECM and ECM-mediated inhibition of viral replication after viral entry into tumor cells. Our observations also suggest that HSV-1 can establish latent infection in some tumor cells present in multicellular spheroids and that this can revert to productive viral infection on outgrowth of individual tumor cells into the Matrigel matrix.

It is well established that 3D tumor cell cultures are useful for preclinical evaluation of the cytotoxic effect of anticancer agents, and multiple cell types within individual tumors have differential sensitivities to drugs and radiation both in vivo and in 3D cultures. Studies have indicated that tumor cells grown in 3D cultures are also more resistant to viral, including HSV-1 and adenovirus-mediated oncolytic therapy, than cells grown in 2D culture. Observations reported here confirm and extend the finding of these studies and identify morphologically distinct tumor cell populations present in 3D cultures that have increased resistance to viral oncolytic therapy. These observations are likely of clinical relevance as, for instance, vasculogenic mimicry patterns are present in a wide variety of malignancies and their detection in several tumor types is associated with adverse outcome.

Theoretical considerations and experimental observations made thus far indicate that mechanisms of tumor resistance to HSV-1 therapy include ECM-mediated impairment of intratumoral virus spread, impaired viral entry into tumor cells because of decreased expression of HSV-1 entry receptors, inhibition of viral replication after viral entry into tumor cells, and virus clearance by the host immune system. In this study, we found that at least two of these potential mechanisms of virus resistance — impaired virus spread in the ECM and inhibition of viral replication after viral entry into tumor cells — are also relevant to 3D tumor cell cultures.

HSV-1 oncolysis can be improved by degradation of fibrillar collagen in tumors indicating that the ECM has an important function in determining treatment efficacy. This study indicates that laminin-rich ECM, Matrigel inhibits HSV-1 spread. It is important to note that HSV-1 could clearly infect and replicate in some uveal melanoma cells that have invaded the Matrigel matrix in this study. These observations indicate that HSV-1 can spread through Matrigel if tumor cells infiltrating the ECM are present.

Interestingly, observations made in this study suggest that HSV-1 establishes quiescent infection in some tumor cells in 3D cultures. When OCM1 and C918 cells were inoculated with HSV-1 under 2D conditions and were then cultured within Matrigel, only a small minority of tumor cells that have taken up HSV-1 showed evidence of virus replication (GFP expression). These findings indicated that the viral replication cycle was inhibited at a post-entry step in the majority of infected tumor cells. Consequent growth of virus inoculated

OCM1 cells in 3D cultures was associated with the establishment of multicellular tumor cell spheroids. Many OCM1 cells forming multicellular spheroids remained GFP negative throughout a 4-week observation period. However, the outgrowth of individual OCM1 cells into the Matrigel matrix from spheroids that have been GFP negative for days to weeks was often associated with the appearance of GFP expression in outgrowing cells and in cells within the spheroids. As Matrigel inhibits virus spread, reappearance of GFP expression in multicellular spheroids was very suggestive of virus reactivation from quiescence in earlier HSV-1 inoculated cells. It is well known that HSV-1 can establish quiescent infection in cultured non-neuronal cells if the progression of the viral replication cycle is blocked shortly after virus entry. However, establishment of quiescent infection in tumor cells induced by the ECM environment and reactivation of viral replication on changing tumor growth pattern have not yet been reported. Our observations reported here provide novel information about virus resistance of distinct tumor cell subpopulations in 3D tumor cell cultures and about mechanisms of ECM-mediated virus resistance of tumor cells.

In summary, to better understand the difficult and compound pathways cooperating with the pathogenesis of HSV-1 the use of tissue and cell cultures and animal models are important, but thus far many issues regarding both pathogenesis and effective therapy are ambiguous. It is clear from this discussion that there are plenty of mentioned topics requiring further exploration with focus on latency to broaden our current knowledge and thereby providing an improved patient care.