1. Introduction

Oncolytic viruses (OVs) directly lyse infected tumor cells, intercepting the cellular transcription of host cells by infection of tumor cells and using a translational mechanism for viral replication.[1] The termination of the viral replication cycle leads to tumor cell lysis and the release of infectious viral progeny.[2] This oncolysis releases viral particles, cell debris, tumor-associated antigens, and cellular damage-associated molecular patterns involved in the immunogenic cell death process.[3] Furthermore, the products of oncolysis, such as cell debris and progeny virions, trigger rapid activation of host antiviral responses that mediate residual infected and uninfected tumor cell destruction.[4] The direct destruction of infected cells can spread to neighboring tumor cells via enabling simultaneous infection of multiple cells in a single infection event.[5] The released progeny virions and cell debris from destroyed cancer cells may spread within surrounding tumor cells or blood vessels, which induces bystander damage of additional tumor cells.[6] Although vessel delivery of replicable oncolytic viral infection is a reasonable assumption, it has not been fully demonstrated.[7] The successful delivery of as many viral particles as possible to the tumor vasculature should be promoted. Virions must escape blood vessels and infect cancer cells. Initial viral infection after intravenous delivery can be confirmed on the periphery of a growing solid tumor.[8] Likewise, spreading of OVs offers the therapeutic opportunity of both primary tumor and any apparent or undiagnosed metastatic deposits simultaneously. The overcome of various barriers for viral oncolysis and the enhancement of the spreading of OVs are a vigorous area for further study.[9] Novel method for effective spreading of OVs to tumor sites may be a very attractive option for the therapy of patients with advanced or metastatic disease.[10] Therefore, a well-defined in vitro model that reflects the spreading of OVs into the vessel, such as occurs in the in vivo microenvironment, can contribute to simulating the assumption of bystander damage[5,11] and OV spreading.[10,12]

To evaluate OVs as anticancer agents, various 3D cell culture methods have been suggested to establish models reflecting...
in vivo-like conditions. In particular, a multicellular tumoroid (MCT) model has been adopted in several studies to mirror the 3D tissue context of in vivo cancer tissue.\[^{13}\]\ As a study using 3D in vitro tumor model, the inhibitory effect of metastasis through infection of OVs in a study of epithelial ovarian cancer metastasis was evaluated by forming 3D tumor spheroids using three types of human ovarian cancer cell lines and infecting various types of OVs.\[^{14}\] However, it was insufficient to be convinced the response of OVs against human tumor tissue by the formation of tumor spheroids with single type cells and the result through direct infection. In tumor microenvironment, there are various components such as extracellular matrix (ECM), resident stromal cells, immune cells and blood flow in tumor microenvironment (TME), which can affect the infectious ability of tumor cells in OVs. Accordingly, it has been reported that an MCT integrated microphysiological system (MPS) effectively provides an in vivo-comparable physiochemical microenvironment, such as chemical gradients and cancer-stromal interactions, contributing to the generation of MCTs and the evaluation of anticancer drugs.\[^{15}\] The MPS with MCTs possessing a cancer microenvironment can help to evaluate anticancer characteristics and spreading of OVs.

Here, we introduce a 3D in vitro MPS, which enables real-time observation of oncolytic infection and spreading of OVs. To identify the spreading and bystander infection of OVs through fluid flow, bystander infection by delivery of OVs was realized by block-to-block linkage of the primary infected MPS with uninfected 3D MCTs integrated with the MPS. MCTs in the MPS were formed using human lung cancer cells (A549), human lung fibroblasts (MRC-5), human umbilical vein endothelial cells (HUVECs), and ECM in this MPS. To verify the oncolytic virotherapy effect due to the spreading of OVs, this study used replicable vesicular stomatitis virus (VSV)-green fluorescence protein (GFP) to identify the location of infection in 3D MCTs that were formed with fluorescence tracker-labeled cells. The spreading of VSV-GFP through fluid flow was identified by tracking GFP expression in 3D MCTs within the connected MPS. Consequently, this device provides a platform for identifying oncoselective infection and the spreading of OVs to distant sites using a connected MPS.

2. Results

2.1. Fabrication of a 3D In Vitro MPS Capable of Verifying Oncolytic Infection by the Spreading of VSV-GFP

Microfluidic device for the MPS was fabricated with the detailed internal dimension shown in Figure S1a, Supporting Information. 3D in vitro MCTs in microfluidic device were formed by the process shown in Figure S1b, Supporting Information. To fabricate a bystander infection system for studying the therapeutic effect of OVs on other target cancers by in vivo vascular structure (Figure 1ai), referred to as the spreading of virus, we designed two MPSs that were connected by block-to-block linkage using a Teflon tube (Figure 1aii). To form the in vitro 3D MCTs in a manner similar to that in the construction of an in vivo tumor microenvironment with vascular flow structure, we performed rapid tumorigenesis of cancer cells with fibroblasts, which affect ECM remodeling, utilizing a microfluidic system established in our previous study.\[^{15}\] Each MPS contained 29 MCTs formed by cancer cells and fibroblasts with type I collagen matrix (Figure 1aiii, white arrowheads). We used these MPSs to perform the bystander infection test according to the medium flow of VSV-GFP from the primary infected MPS to the linked MPS for delivery of virus. This bystander infection system was designed to allow the delivery of VSV-GFP toward a linked MPS by medium flow through a tube connection, similar to the spreading of OVs by the in vivo blood flow (Figure 1aiii).

Viral infection of VSV-GFP within the MPS containing the 3D MCTs, which were formed by red fluorescence tracker-labeled cells (Figure S2, Supporting Information), was performed by flow through the inlet hole of the MPS using a microtip at 5 MOI (multiplicity of infection). The residual VSV-GFP in the MPS was removed after incubation for 1 h (Figure S3i,ii). The removed VSV-GFP was analyzed to quantify the residual VSV-GFP after infection. As a result, the quantification analysis indicated substantial infection of the MCTs in the MPS at ≈1 MOI. This primary infected MPS was connected with both a passive micropump to supply continuous medium and a drain reservoir and was incubated for 24 h (Figure 1bi and Figure S3iii, Supporting Information). After incubation of the primary infected MPS for 24 h, this chip was linked with an MPS containing non-fluorescence-labeled 3D MCTs for the bystander infection test and incubated for 48 h at 37 °C and 5% CO₂ (Figure 1bii and Figure S3iv, Supporting Information).

2.2. Verification of the Oncoselective Infection via the Spreading of VSV-GFP within the Linked MPS

GFP expression due to VSV-GFP infection appeared in the oncoselectivity in the cancer cell region of MCTs formed with red fluorescence labeling (Figure S2, Supporting Information) in the primary infected MPS but not in the fibroblast region (Figure S2, Supporting Information). GFP expression by VSV-GFP infection showed a high level of fluorescence intensity at PI 24 h and then a post-infection (PI) time-dependent decrease. In addition, GFP expression was partially confirmed in the bystander MPS at 24 h after the bystander infection was detected by identifying a PI-dependent increase in the G-F expressing area in MCTs of the linked chip (Figure 2). This result demonstrates that the virion of VSV-GFP released after direct infection in the primary MPS delivered to the bystander MPS through spreading via flow condition in the link system.

2.3. Verification of the Oncolytic Effect by the Spreading of VSV-GFP

To identify cell death as oncolytic cytopathogenesis by the spreading of VSV-GFP, MCTs formed with non-fluorescence-labeled cells were analyzed by live/dead staining to detect the area of dead cells overlapping with the GFP-expressing area after VSV-GFP infection by spreading. The near overlap of the VSV-GFP-infected location and the dead cell area was evaluated in the stained images. Specifically, dead cells were not identified.
in the region where fibroblasts were present in MCTs. The oncolytic effect of the bystander infection of VSV-GFP was also verified by confirming that a similar pattern was also observed in the linked MPS. In addition, the area of dead cells in the bystander infected MPS also increased along with PI time (Figure 3a, Figure S4, Supporting Information). In the analysis of the VSV-GFP expression area and dead cell area, the VSV-GFP area showed a PI time-dependent decrease (Figure S4, Supporting Information), and the dead cell area increased at PI 48 h but decreased at 72 h. In the bystander infected MPS, this percentage showed a PI time-dependent increase (Figure 3b). These results demonstrate the oncolysis effect of the spreading of VSV in fold change analysis of the dead cell area compared to the VSV-GFP area; a score of 1.0 = 100% cell death due to VSV infection (Figure 3c).

To evaluate a change of the tumor growth due to the spreading of VSV-GFP within the MPS, the MCT area was continuously monitored for 72 h after infection (Figure S5, Supporting Information). The results showed that the area (~140–110 µm²) of infected MCTs gradually decreased in contrast to the increasing area (~170–210 µm²) of noninfected MCTs as a control. In addition, it was observed that the growth of the MCT area was inhibited after linkage of MPSs for spreading (Figure 3d). The change in MCT area was significantly different according to PI time or experimental group.

2.4. PI Time-Dependent Change in Viral Quantification in the Linked MPS for the Spreading of VSV-GFP

Oncolytic infection of VSV-GFP was assumed to promote the enhancement of replicability and spreading to other tumor sites. Therefore, to verify washing of VSV-GFP by the fluid flow system and the quantitative changes of replicable VSV-GFP due
to spreading, fluorescence intensity and TCID analysis were used. The PI time-dependent reduction in VSV-GFP expression intensity in the no-link system was confirmed, and the same pattern was identified in the primary infected chip of the link system for spreading. However, the result of verifying the spreading by the linked MPSs was an increase in VSV-GFP fluorescence intensity in the linked chip (Figure 4a).

To evaluate whether the linkage between MPSs triggers a change in VSV-GFP production, we performed TCID analysis using the elute that was collected at PI 24, 48, and 72 h. The elute of the no-link system was collected from the drain reservoir at PI 24, 48, and 72 h. The collection schedule of the elute for TCID analysis was performed according to the time procedure shown in Figure S6, Supporting Information. As a result of comparison analysis of infectious virus particles in each group of both the no-link system and the link system with two-way RM ANOVA, the time-dependent steady increase in VSV-GFP was quantified. There was a significant difference at PI 72 h between the no-link system and link system, with p < 0.01 (Figure 4bi). Furthermore, the increase (>fivefold) in VSV-GFP quantification for the linked MPSs was obviously assessed by the total quantification analysis of VSV-GFP in comparison with the no-link system, and the amount for the link system at PI 72 h increased to the amount at PI 24 h for the no-link system, as shown in Figure 4ci. Although the viral genome detection results were not different at PI 48 and 72 h for the link system, the overall pattern showed a similar tendency with the results for infectious virus particles (Figure 4ci). The similar tendency provided a higher amount of virus for the link system than for the no-link system, according to the analysis of the total viral genomes (Figure 4cii). Quantitative results of the overall viral genome showed a pattern similar to the tendency identified in the TCID results.

In these results, it was demonstrated that the delivery of VSV-GFP particles, called spreading, occurred from the primary infected MPS to the bystander MPS by fluid flow in this link system. The infected MCTs produced large amounts of IPs. This 3D in vitro MPS provides the possibility to simulate the spreading of this virus.

2.5. PI Time-Dependent Change in Expression of the Cytoplasmic VSV-Related Proteins within the 3D In Vitro MPS

After infection with VSV-GFP, immunofluorescence staining analysis was performed to identify PI time-dependent changes in VSV-related proteins. As a priority, the expression of both VSV-glycoprotein (VSV-G) and interferon-beta (IFNβ) in MCTs under 3D in vitro microfluidic conditions was confirmed before
**Figure 3.** Cell death by oncolytic infection and bystander infection of VSV in the MPS with the link system. a) The oncolytic effect of the bystander infection of VSV-GFP was also assessed by confirming that a similar pattern was also observed in the linked MPS. b,c) PI time-dependent increase of dead cells area in the bystander infected MPS; Fold score of 1.0 = 100% cell death due to VSV infection. d) The change of MCT area to evaluate the oncolytic effect of VSV-GFP on tumor growth for 72 h after infection. **p < 0.01, *p < 0.05.

**Figure 4.** The change in VSV quantification was dependent on PI time in the link system of MPSs. a) PI time-dependent reduction of VSV-GFP expression intensity in the no-link system and the link system for bystander infection. b) TCID_{50} analysis to evaluate whether the linkage between MPSs triggers a change in VSV production using the elute (i) VSV production by PI time, ii) Total production of VSV. c) Total quantification analysis of VSV in comparison on both the no-link and the link system of MPSs (i) Viral genome of VSV by PI time, ii) Total viral genome of VSV. **p < 0.01, *p < 0.05.
VSV-GFP infection. VSV-G is a surface molecule of VSV-GFP that is activated after interaction with cancer cells and IFNβ is considered to may be activated after infection in IFN signaling deficiency cancer cells. The expression of all VSV-related proteins was not detected without VSV-GFP infection (Figure S7, Supporting Information). After infection in this 3D in vitro MPS, the expression of VSV-G at PI 24 h almost completely overlapped with the cancer cell regions of MCTs with high GFP expression by VSV-GFP (Figure 5ai and Figure S8, Supporting Information). The location of expression of IFNβ was also similar to that of VSV-G expression (Figure 5a(ii) and Figure S9, Supporting Information). However, the pattern of decreased expression of all these proteins showed a PI time-dependent that was the same as that of the decrease in GFP expression by VSV-GFP at PI 72 h (Figure 5ai,ii images and Figures S8 and S9, Supporting Information). Analysis of changes in the expression areas of both VSV-G and IFNβ over time after infection clearly shows these sequential PI time-dependent fluorescence decreases (Figure 5ai,ii graphs).

The expression area of all proteins in MCTs of the primary infected chip at PI 72 h was compared with the expression area in the bystander MPS infected by the spreading of VSV-GFP (Figure 5bi,ii capture images and Figure S10, Supporting Information). Differences in the degree of VSV-GFP infection in MCTs in the bystander system resulted in large variation in the error bars in the expression area of all proteins and GFP expression area compared to those in the primary infected MPS, but significant expression of the related proteins by bystander infection through spreading of VSV-GFP was assessed (Figure 5bi,ii graphs).

2.6. Analysis of Human Interferon-Beta Production as an Immune Response to VSV-GFP Infection within 3D In Vitro MPSs

To analyze the stimulation pattern of the innate immune response induced by detecting VSV-GFP in 2D culture conditions of each cell line constituting 3D MCTs, an ELISA for human IFNβ was performed to measure secreted IFNβ quantitatively. VSV-GFP was inoculated at 1 MOI on each cell line prepared in a 6-well plate, and the supernatant was collected at PI 6, 14, and 24 h. An ELISA was then carried out. It was verified that A549 cells, MRC-5 cells, and HUVECs showed a different pattern of IFNβ secretion in response to VSV-GFP infection. Regardless of time, A549 cells showed low secretion of IFNβ. HUVEC IFNβ secretion was lower than that of A549 cells at PI 6 h and showed an increasing pattern of IFNβ secretion at
PI 14 and 24 h. However, MRC-5 cells secreted IFNβ at a high level from 6 to 14 h after VSV-GFP infection. This result shows that the IFNβ secretion pattern corresponding to VSV-GFP infection in the 2D culture condition is different depending on the type of cell, and VSV-GFP, which is sensitive to IFN signaling, can utilize this characteristic for oncolytic infection as an OV (Figure S11a, Supporting Information).

To assess IFNβ expression in 3D conditions, a similar analysis was performed using the 3D in vitro MPS to compare the patterns of human IFNβ secretion between the no-link system and the link system. VSV-GFP was inoculated on 3D MCTs within the MPS at 5 MOI for 1 h. The drained elutes were collected at PI 6, 14, and 24 h and used as ELISA samples for the no-link system. Then, an uninfected MPS was connected at 24 h after infection, and the drained elute at the same time point was collected and used as a sample of the link system to perform an ELISA for secreted IFNβ. Analysis of the secreted IFNβ in the no-link system was not sensitive at all time points, but it was identified that the IFNβ secretion was detectable at PI 14 to 24 h in the link system although there was a quantitative variation. The amount of IFNβ secreted by the immune response in the 3D in vitro MPS was higher than that in the no-link system (Figure S11b, Supporting Information).

In addition, to quantitatively identify the gene-level expression of human IFNβ between the no-link system and the link system, mRNA was extracted from lysates of the 3D MCTs at the same time as the time as the IFNβ secretion analysis. These mRNAs were amplified to cDNA via quantitative RT-PCR, and cDNA was used to identify the expression of the IFNβ gene. The expression of the IFNβ gene in the no-link system tended to increase continuously until 24 h after VSV infection, suggesting that the immune response is activated. In addition, the expression of the IFNβ gene in the link system, a model between the primary infected MPS and the uninfected, was found to be higher than that in the no-link system at 14 h (link system PI 14 h) (Figure 6). Data from both ELISA and gene expression analysis between the no-link system and the link system showed similar trend patterns and these results demonstrate that viral infection was caused by the spreading of VSV to the bystander MPS through the link system. This 3D in vitro MPS has proven to be a model capable of spreading to other cancer cells, the second target of replicable OVs.

3. Discussions

Current therapies, such as radiotherapy or chemotherapy, to conquer cancer are often limited in their effectiveness, so a new approach for cancer therapy is the use of viruses.[16] The delivery and penetration of viruses into solid tumors to mediate cancer therapy efficacy confronts a series of obstacles. To date, most gene therapy strategies use nonreplicating viruses because of the risk of uncontrolled viral spreading when replicating viruses are administered to a patient.[17] However, if the goal is to eliminate transfected cells such as cancer cells, the unique cytotoxic characteristics of viral replication can be advantageous. Nonreplicating viruses can be deposited only through direct injection or the vasculature, while replicating viruses have the ability to spread in a tumor, which can infect a substantial number of targeted cancer cells. Virus replication is usually rapid, and cell lysis is usually completed within 7 h of infection.[16] Because of the heavy biochemical demands that these replicating viruses produce for replication in host cells, apoptotic cell death can often occur.[18] At the completion of the viral replication cycle, many viruses induce lysis of host cells, transferring newly synthesized viral particles to other cells.[19] In the tumor microenvironment (TME), viral replication also induces innate and adaptive immune reactions that limit viral spread, and the presence of virus together with cell lysis, tumor antigens, and related molecular patterns can overcome immunosuppression and promote antitumor immunity.[23] Therefore, use of replicating OVs instead of nonreplicating viruses to conquer cancer leads to a novel mode of cancer therapy called oncolytic virotherapy.

The spreading of OVs provides the opportunity to simultaneously treat both the primary tumor and any metastatic deposits. Regarding fabrication of an in vitro platform capable of evaluating this spreading, we designed an MPS via a link system of two microfluidic-based MPSs to allow the delivery of OVs to metastases or any cancer deposits through in vivo-like dynamic conditions, such as those in the bloodstream. The flow condition used in the MPS is similar to flow level that can generate the interstitial flow and shear stress for simulating the in vivo microenvironment.[20] Although this MPS simply mimics the microenvironment such as capillary flow without several factors including immune and blood cells which needed to completely mimic the in vivo tissue microenvironment, it could simulate the OV delivery like in vivo blood flow which affects the replication and spreading of OV. Among OVs used to evaluate spreading, VSV is a suitable OV that has the merit of rapid growth to high titers in a broad range of cell lines.
facilitating large-scale virus production.[13] The bystander infection by VSV-GFP spreading within our MPS was confirmed by the GFP expression in MCTs of the linked MPS. In this process, delivery of VSV-GFP-infected cells was not observed. It can be concluded that in this bystander infection, VSV-GFP migrated from the MPS containing the primary infected MCTs to the MCTs containing any MCTs through spreading by fluid flow. This MPS enabled the real-time monitoring of the oncosensitivity and spreading with fluorescence-labeled OVs.

It was also considered that replication of the VSV-GFP occurred by confirming that the GFP expression showed a time-dependent decrease in the primary infected MPS, while the GFP expression showed a time-dependent increase in the bystander infected MPS. Consequently, we predicted that viral titers might increase with more stable replication when there is any site that could be harboring a viral infection. To evaluate the stable replication of VSV-GFP after bystander infection in the link system, the elute in the drain reservoir was used to quantify viral titers compared with those in the no-link system. As a result, we obtained the same results as expected, which suggests that the link system of MPSs is able to evaluate the spreading of OVs.

After infection of 3D MCTs with VSV-GFP in this MPS, we compared the process of virus infection and replication through the expression of the viral protein VSV-G and the subsequent innate immune response between the no-link and link systems. The distribution of both VSV-G and IFNβ expression showed signal patterns with time-dependent changes similar to those for GFP. This result shows the oncolytic effect by rapid replication of VSV in tumor cells[21] and demonstrates that suppressed type I IFN signaling is caused by a broken IFNβ secretion pathway in which IFNβ is not translated in A549 cancer cells. At 72 h PI in the link system of MPSs, locations of GFP, VSV-G, and IFNβ expression in the primary infected MPS overlapped, similar to that of the no-link system. Additionally, due to fluid flow with a constant velocity, the fluorescent area of every signal in the bystander infected MPS was observed to be similar to that of the primary infected MPS. This microfluidic platform-based MPS provides similar conditions to 3D MCTs of the linked MPS by spreading VSV, an OV. Moreover, these results indicate that VSV is a promising OV for spreading, as has been revealed in several studies.[7,13a,22]

To analyze the stimulation pattern of the innate immune response induced by detecting VSV-GFP in 2D culture conditions of each cell line constituting 3D MCTs, we performed an ELISA for human IFNβ to measure secreted IFNβ quantitatively. VSV-GFP was inoculated at 1 MOI, and the supernatant was collected at PI 6, 14, and 24 h. An ELISA was then carried out. It was verified that A549 cells, MRC-5 cells, and HUVECs showed a different pattern of IFNβ secretion in response to VSV-GFP infection. The patterns of IFNβ secretion when each cell line was infected with VSV in 2D culture conditions were interesting. By substituting this ELISA result into the experimental result of observing the viral sensitivity of cells in 2D and the IFNβ secretion aspect, the amount of IFNβ secreted from MRC-5 fibroblasts was high at the initial time point after infection, and MRC-5 cells were protected from VSV infection, with low viral sensitivity. Furthermore, it was detected that the sensitivity of HUVECs was significantly different from that of MRC-5 cells, as a clear difference in IFNβ secretion in response to VSV infection was observed. This result shows that the IFNβ secretion pattern corresponding to VSV-GFP infection in the 2D culture condition is different depending on the type of cell, and VSV-GFP, which is sensitive to IFN signaling, can utilize this characteristic for oncolytic infection as an OV. Then, we examined the pattern of IFNβ secretion in the 3D in vitro MPS, comparing the no-link and link systems. In the no-link system, detection of secreted IFNβ was difficult because the total cell numbers in the 3D MCTs were much lower than that in the 2D condition. Unlike this result, in the link system of MPSs that had more cell numbers in the 3D MCTs, detection of secreted IFNβ was conveniently possible using drained elute. We found that the secreted IFNβ from the linked chip was measurable because of the additional bystander infection of 3D MCTs via fluid carrying infectious VSV, which led to additional IFNβ being secreted from the infected 3D MCTs. Therefore, this problem can be solved through the connection of more MPSs. Accordingly, we demonstrated that this 3D in vitro MPS has usefulness, as the patterns of various secreted cytokines related to the immune response induced by infection in the TME can be easily analyzed when applied to cancer therapeutic research for OV spreading. As a result, this MPS enabled to observe the oncolytic property of OVs in real time and to evaluate the molecular activity and to identify the delivery effect by OVs spreading on flow condition.

4. Conclusion

We evaluated the spreading of OVs using a fluid flow integrated 3D in vitro MPS capable of identifying OVs as anticancer agents. By utilizing the link system of MPSs, including the in vivo-like dynamic microenvironment, the bystander OV infection of other target cancer cells was assessed by VSV-GFP spreading through fluid flow similar to that in blood flow in vivo through the vascular structure. This MPS enabled non-invasive real-time monitoring of oncolytic activity, such as oncoslectivity and spreading, and the characterization of OVs through various analytical methods. We expect that this 3D in vitro MPS will be useful for studying oncotarget delivery using various types of OVs and cancers as a system that can evaluate spreading and immune-related responses induced by viral infection.

5. Experimental Section

Production of the MPS and Fabrication of a Passive Powerless Micropump: In the previous study, the fabrication procedure of the polydimethylsiloxane (PDMS; Sylgard 184, Dow Chemical Co., MI, USA)-based microfluidic device is described.[15] Cured fluidic chips were pulled out from a mold, and then inlet and outlet holes were made using a 2 mm diameter tissue punch. Sterilized microfluidic devices and cover glass were bonded after treatment with oxygen plasma (Femto Science, Korea). Then, fabrication and verification of the microfluidic system using a passive micropump with a siphon effect was established in the previous studies.[15, 26]
Cell Culture and Generation of the In Vitro 3D MCT Model: The A549 (a human lung adenocarcinoma cell line, ATCC; CCL-185, Manassas, VA, USA) and MRC-5 (a human lung fibroblast cell line, ATCC; CCL-171) cell lines were cultured in DMEM (HyClone, MA, USA) and Dulbecco's modified Eagle's medium (ThermoFisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Biowest, MO, USA) and 1% antibiotics (ThermoFisher Scientific). Human umbilical vein endothelial cells (HUVECs; ATCC; CRL-1730) were grown in endothelial cell medium (ECM; Sciencell Research Laboratories Inc., CA, USA) consisting of 500 mL of basal medium, 5% FBS, 20 ng mL\(^{-1}\) VEGF, and 1% penicillin/streptomycin solution. The pH level of prepolymerized collagen solutions was maintained in vivo condition, ranging from 7.4 to 8.0. It was ensured that the pH of the prepolymerized collagen solution reached 7.4-8.0. To generate an in vitro tumor model, the micro-wells of the microfluidic system were coated with a 2 mg mL\(^{-1}\) type I collagen solution (BD Bioscience, San Jose, CA, USA) using a micropipette, and the collagen solution was removed by aspiration. A549 and MRC-5 cells (2.0 \(\times\) 10\(^6\) cells mL\(^{-1}\)) were each suspended in a 2 mg mL\(^{-1}\) type I collagen solution. A549 cells were gently injected into the microfluidic system through the inlet hole, and the device was centrifuged at 6000 revolutions per minute (RPM) for 30 s using a minicentrifuge (KA. MC-01; Korea Ace Scientific, Seoul, Korea) to trap the cancer cells in the microwell. After aspiration of the remaining cancer cells, the device was centrifuged at 6000 RPM for 30 s using a minicentrifuge and aspirated. The microfluidic system was rinsed with a collagen injection and aspirated three times. It was then incubated at 37 °C for 30 min for gelation of the collagen in the microwell. After incubation for 30 min, HUVECs (1.0 \(\times\) 10\(^6\) cells mL\(^{-1}\)) were seeded in a microfluidic channel to generate a vessel-like environment and incubated for 3 h at 37 °C so that they adhered to the vessel-like surface. Following cell attachment, the microfluidic system was connected to a passive micropump established in the previous study\(^{[15,20]}\). The formation of MCTs was monitored daily using a phase-contrast microscope (EVOS; Life Technologies, Carlsbad, CA, USA).

Generation of VSV-GFP as a Recombinant VSV: The VSV full-length plasmid pVSV-Venus-VSVG (Addgene, Watertown, Massachusetts, USA) was the template to generate VSV-eGFP. It was generated as previously described\(^{[21]}\). Briefly, 293T cells were plated in 6-well plates at a density of 1 \(\times\) 10\(^6\) cells per well. The cells were infected with vaccinia virus encoding T7 polymerase (vTF7-3; ATCC) at 10 MOI. After 1 h, the residual virus was washed, and the cells were transfected with 1.1 g of pN, 1.4 g of pP, and 0.9 g of pVSV-Venus-VSVG at 5 MOI. After 1 h of infection, the device was centrifuged at 6000 RPM for 30 s using a minicentrifuge and aspirated. The microfluidic system was rinsed with a collagen injection and aspirated three times. It was incubated at 37 °C for 30 min for gelation of the collagen in the microwell. After incubation for 30 min, HUVECs (1.0 \(\times\) 10\(^6\) cells mL\(^{-1}\)) were seeded in a microfluidic channel to generate a vessel-like environment and incubated for 3 h at 37 °C so that they adhered to the vessel-like surface. Following cell attachment, the microfluidic system was connected to a passive micropump established in the previous study\(^{[15,20]}\). The formation of MCTs was monitored daily using a phase-contrast microscope (EVOS; Life Technologies, Carlsbad, CA, USA).

Virus Growth Kinetics: The 3D MCTs in the MPSs were infected with VSV-GFP at 5 MOI. After 1 h of infection, the chips were washed with PBS, and fresh ECM (Sciencell Research Laboratories Inc.) was supplied. The drained elute from the infected chips was collected at 24, 48, and 72 h for measurement of infectious viral particles by TCID\(_{50}\), as described previously\(^{[16]}\).

Statistical Analysis: The area and fluorescence intensity of the MCTs were determined by ImageJ software (ver 1.46, NIH, USA). The number of samples for analysis is indicated in each data figure. Quantitative data are presented as the mean ± SD. Group differences were assessed by paired t-tests or one-way and two-way ANOVA using ORIGIN 2018b (OriginLab Corp., MA, USA). Statistical significance was set at p < 0.05 (ns), p < 0.05 (*), p < 0.01 (**), and p < 0.005 (***).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF-2019R1A2C2005244 to G.S.J.; NRF-2019R1C1C1007468 to K.J.L.).

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

S.W.L. and K.J.L. contributed equally to this work. S.W.L. contributed to the study conception and design, administrative support, data collection and assembly, data analysis and interpretation, and manuscript writing; K.J.L. contributed to the study conception and design, financial support, administrative support, data collection and assembly, data analysis and interpretation, and manuscript writing; S.Y. and C.H.J. contributed to the data analysis and interpretation and manuscript writing; H.R.L. contributed to the study conception and design, provision of study materials, critical drafting and revision of the work for important intellectual content, and final approval of the manuscript; and G.S.J. contributed to the study conception and design, financial support, provision of study materials, critical drafting and revision of the work for important intellectual content, and final approval of the manuscript.

Keywords

drug discovery, microfluidic platform, microphysiological system, oncolytic virus, preclinical study

Received: July 8, 2019
Revised: October 4, 2019
Published online: December 11, 2019
[1] a) M. J. Vaha-Koskela, J. E. Heikkila, A. E. Hinkkanen, Cancer Lett. 2007, 254, 178; b) D. E. Dorer, D. M. Nettelbeck, Adv. Drug Delivery Rev. 2009, 61, 554.
[2] a) A. E. Tollefson, A. Scaria, T. W. Hermiston, J. S. Ryerse, L. J. Wold, W. S. Wold, J. Virol. 1996, 70, 2296; b) H. Jiang, E. J. White, C. I. Rios-Vicil, J. Xu, C. Gomez-Manzano, J. Fueyo, J. Virol. 2011, 85, 4720.
[3] A. C. Filley, M. Dey, Front. Oncol. 2017, 7, 106.
[4] a) H. L. Kaufman, F. J. Kohlhapp, A. Zloza, Nat. Rev. Drug Discovery 2015, 14, 642; b) B. D. Lichty, C. J. Breitbach, D. F. Stojdl, J. C. Bell, Nat. Rev. Cancer 2014, 14, 559.
[5] L. Sprague, L. Braidwood, J. Conner, K. A. Cassady, F. Benencia, T. P. Cripe, Future Virol. 2018, 13, 671.
[6] E. Smith, J. Breznik, B. D. Lichty, Hum. Gene Ther. 2011, 22, 1053.
[7] S. J. Russell, K. W. Peng, J. C. Bell, Nat. Biotechnol. 2012, 30, 658.
[8] C. J. Breitbach, J. M. Paterson, C. G. Lemay, T. J. Falls, A. McGuire, K. A. Parato, D. F. Stojdl, M. Daneshmand, K. Speth, D. Kirn, J. A. McCart, H. Atkins, J. C. Bell, Mol. Ther. 2007, 15, 1686.
[9] D. G. Roy, J. C. Bell, Oncolytic Virol. 2013, 2, 47.
[10] M. S. Ferguson, N. R. Lemoine, Y. Wang, Adv. Virol. 2012, 2012, 805629.
[11] J. Ady, V. Thayanithy, K. Mojica, P. Wong, J. Carson, P. Rao, Y. Fong, E. Lou, Mol. Ther.–Oncolytics 2016, 3, 16029.
[12] J. Maroun, M. Munoz-Alia, A. Ammayappan, A. Schulze, K. W. Peng, S. Russell, Future Virol. 2017, 12, 193.
[13] a) E. Fennema, N. Rivron, J. Rouwkema, C. van Blitterswijk, J. de Boer, Trends Biotechnol. 2013, 31, 108; b) J. Friedrich, C. Seidel, R. Ebner, L. A. Kunz-Schughart, Nat. Protoc. 2009, 4, 309.
[14] J. G. Tong, Y. R. Valdes, J. W. Barrett, J. C. Bell, D. Stojdl, G. McFadden, J. A. McCart, G. E. DiMattia, T. G. Shepherd, Mol. Ther.–Oncolytics 2015, 2, 15013.
[15] S. W. Lee, H. S. Kwak, M. H. Kang, Y. Y. Park, G. S. Jeong, Sci. Rep. 2018, 8, 2365.
[16] J. Goldufsky, S. Sivendran, S. Harcharik, M. Pan, S. Bernardo, R. H. Stern, P. Friedlander, C. E. Ruby, Y. Saenger, H. L. Kaufman, Oncolytic Virother. 2013, 2, 31.
[17] S. Bais, E. Bartee, M. M. Rahman, G. McFadden, C. R. Cogle, Adv. Virol. 2012, 2012, 186512.
[18] H. Everett, G. McFadden, Trends Microbiol. 1999, 7, 160.
[19] M. T. Madigan, J. M. Martinko, J. Parker, Brock Biology of Microorganisms, Prentice-Hall, Inc., Upper Saddal River, NJ 2000.
[20] G. S. Jeong, J. Oh, S. B. Kim, M. R. Dokmeci, H. Bae, S. H. Lee, A. Khademhosseini, Lab Chip 2014, 14, 4213.
[21] J. H. Connor, C. Naczki, C. Koumenis, D. S. Lyles, J. Virol. 2004, 78, 8960.
[22] K. M. Yamada, E. Cukierman, Cell 2007, 130, 601.
[23] J. B. Ruedas, J. H. Connor, Methods Mol. Biol. 2017, 1581, 203.
[24] D. D. LaBarre, R. J. Lowy, J. Virol. Methods 2001, 96, 107.