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The influence of oncolytic reovirus on the biological activities of umbilical cord-derived mesenchymal stem cells

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Abstract. – OBJECTIVE: To investigate the influence of oncolytic reovirus on the biological activities of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) as a novel virotherapy strategy.

MATERIALS AND METHODS: The Cell Counting Kit-8 assay was used to detect the viability of hUC-MSCs infected with different multiplicities of infection (MOIs) of reoviruses. The biological activities (proliferation, marker expression, multipotency, and migration) of hUC-MSCs were verified by assaying osteogenic and adipogenic differentiation potential, flow cytometry, and electrical cell-substrate impedance sensing, respectively.

RESULTS: The viability of hUC-MSCs slightly decreased by infection with low titers of reoviruses. A MOI of 1 had no effect on the viability of hUC-MSCs within 96 h. The biological activities (proliferation, marker expression, multipotency, and migration) of hUC-MSCs were not affected by reovirus infection at a MOI of 1.

CONCLUSIONS: Reovirus at a MOI of 1 had no effect on the biological activities of hUC-MSCs.

Key Words:
Oncolytic reovirus, Umbilical cord-derived mesenchymal stem cells, Proliferation, Differentiation, Migration.

Abbreviations

CD, cluster of differentiation; ECIS, electrical cell-substrate impedance sensing; hUC-MCSs, human umbilical cord-derived MSCs; MOI, multiplicity of infection; MSCs, mesenchymal stem cells.

Introduction

Despite advances in targeted therapies and cellular immunotherapies, there has been no fundamental improvement in cancer patients' overall survival rate. The mechanism and efficacy of oncolytic viruses for the treatment of tumors have been of widespread interest and the focus of basic and clinicians' studies. Oncolytic viruses are non-pathogenic viral particles that infect and kill tumor cells, but have no evident toxicity or tumorigenicity to normal tissues[1,2]. To date, various oncolytic viruses, including adenovirus, measles virus, reovirus, herpes simplex virus, Newcastle disease virus, vesicular stomatitis virus, and vaccinia virus, have been investigated for use in cancer therapies[3]. Of these oncolytic viruses, wild-type reovirus has oncolytic effects, which makes it useful in basic and clinical research.

Reoviruses were first isolated from the respiratory and enteric tracts in the 1950s[4]. Reovirus infections are usually asymptomatic, as the immune response of most people produces neutralizing antibodies[5]. Selective replication and cytolysis of reoviruses are related to the activation of the RAS signaling pathway, constitutively activated in many human tumors. Reovirus replicates and accumulates continuously in tumor cells, which eventually leads to cell lysis and the release of a large number of viruses[6,7]. In addition, reovirus promotes the secretion of inflammatory cytokines and chemokines, triggers a series of immune re-

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sponses to reverse the tumor-induced immunosuppressive microenvironment, and promotes anti-tumor immune responses[8,9].

Phase I, II, and III clinical trials using reoviruses have been initiated to explore treatments for a variety of tumor types (www.clinicaltrials.gov). To date, most reovirus-based therapies have been administered intravenously (Table I). B lymphocytes produce neutralizing antibodies to hamper the spread of reoviruses, while oncolytic virus-neutralizing antibodies are common in most adults. Therefore, the presence of neutralizing antibodies reduces the transduction of tumor cells. However, nonspecific binding of reoviruses to blood cells, uptake by non-target tissues and organs (spleen, lung, and liver), and host production of complement and pre-immune immunoglobulin M hamper the delivery of therapeutic viruses[10,11]. Therefore, overcoming the destructive effect of neutralizing antibodies to reoviruses is crucial to enhance oncolytic virus-based therapies' efficacy.

Virus-shielding strategies to improve viral transfer to tumors have been explored. One strategy involves the use of cellular delivery vehicles. Appropriate carrier cells can shield viruses from neutralizing antibodies, thereby ensuring the biological activities of viruses and cells, and the ability to target tumors and the tumor matrix[12,13]. A number of cell types have been investigated as oncolytic virus delivery vehicles, including bone marrow cells, macrophages, lymphocytes, and endothelial cells[12,14,15]. However, many potential candidate carrier cells are technically difficult to isolate and culture. Hence, the aim of the present study was to explore the influence of reoviruses on the biological activities of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) by analyzing cell viability, proliferation, marker expression, adipogenic and osteogenic differentiation, and migration.

Materials and Methods

Cell Lines and Reovirus

Mouse L929 fibroblasts were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and preserved in the Tissue Engineering and Stem Cell Experimental Center of Guizhou Medical University (Guiyang, China). The L929 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Gaithersburg, MD, USA), and maintained at 37°C under a humidified atmosphere of 5% CO_2/95% air in an incubator. Reoviruses were kindly provided by Professor Xing Zhao (Tissue Engineering and Stem Cell Experimental Center, Guizhou Medical University).

Isolation of MSCs From Human Umbilical Cords and Culturing

The study protocol was approved by the Institutional Review Board of Guizhou Medical University, and written informed consent was obtained from all donors. hUC-MSCs were isolated from three full-term healthy infants delivered by Caesarean section (gestation age, 39-40 weeks) and expanded *in vitro* as previously described[16,17].

Reovirus Production

L929 cells were infected with reovirus at a multiplicity of infection (MOI) of 3 and collected when approximately 60-70% of the cells stained positive with Trypan Blue[18]. Cell pellets were resuspended in virus stabilization buffer (150 mM NaCl, 15 mM $MgCl_2$, 10 mM Tris, pH 7.4), freeze-thawed three times, and stored at -80°C. The median tissue culture infective dose of the L929 cells was determined as described previously[19].

Reovirus Infection of hUC-MSCs

The hUC-MSCs were treated with a reovirus-containing culture medium or mock medium for 2 h. Cells were washed with DMEM/F-12 medium (Gibco, Gaithersburg, MD, USA) and cultured in growth medium.

CCK8 Assays

Cell viability was determined using the CCK8 assay (Dōjindo Laboratories, Kumamoto, Japan). The hUC-MSCs were plated in the wells of 96-well plates at 10^3 cells/well. The next day, cells were infected with reovirus at the indicated MOIs and cultured in 100 µL of growth medium and then incubated with 10 µL of CCK8 solution after culturing in growth medium for the indicated times. After a 2-h of incubation period, cell viability was quantified by measuring absorbance at 450 nm. Measurements were in performed triplicate for each experiment and all experiments were repeated three times.

Flow Cytometry Analysis of hUC-MSC Marker Expression

The hUC-MSCs were identified using a Beckman FC500 MCL flow cytometer (Beckman

Table I. Clinical trials of anti-tumor therapies with the use of oncolytic reoviruses.

NCT Number	Status	Conditions	Interventions	Route	Phases
NCT02444546	Active, not recruiting	Childhood Astrocytoma, Childhood Atypical Teratoid, Rhabdoid Tumor, Diffuse Intrinsic Pontine Glioma, Glioma, Recurrent Childhood Anaplastic Oligodendroglioma, Recurrent Childhood Brain Neoplasm, Recurrent Childhood Glioblastoma, Recurrent Childhood Medulloblastoma, Recurrent Primitive Neuroectodermal Tumor, Refractory Brain Neoplasm	Wild-type Reovirus, Sargramostim	IV	1
NCT01240538	Completed	Unspecified Childhood Solid Tumor, Protocol Specific	wild-type reovirus, cyclophosphamide	IV	1
NCT03282188	Withdrawn	Melanoma, Cancer of Skin	Reolysin, GM-CSF	IV	1,2
NCT01199263	Active, not recruiting	Recurrent Fallopian Tube Carcinoma, Recurrent Ovarian Carcinoma, Recurrent Primary Peritoneal Carcinoma	Paclitaxel, Pelareorep	IV	2
NCT00651157	Completed	Recurrent Melanoma, Stage IV Melanoma	Wild-type reovirus	IV	2
NCT01274624	Completed	KRAS Mutant Metastatic Colorectal Cancer	REOLYSIN, Irinotecan, Leucovorin, Fluorouracil (5-FU), Bevacizumab	IV	1
NCT01280058	Completed	Pancreatic Acinar Cell Carcinoma, Pancreatic Ductal Adenocarcinoma, Recurrent Pancreatic Carcinoma, Stage IV Pancreatic Cancer	Wild-type Reovirus, Carboplatin, Paclitaxel	IV	2
NCT00861627	Completed	Carcinoma, Non-small Cell Lung	REOLYSIN, Carboplatin, Paclitaxel	IV	2
NCT00602277	Completed	Recurrent Fallopian Tube Carcinoma|Recurrent Ovarian Carcinoma|Recurrent Primary Peritoneal Carcinoma	Wild-type Reovirus	IV	1
NCT02514382	Active, not recruiting	Recurrent Plasma Cell Myeloma, Refractory Plasma Cell Myeloma	Wild-type Reovirus, Bortezomib, Dexamethasone	IV	1
NCT03605719	Recruiting	Recurrent Plasma Cell Myeloma	Wild-type Reovirus, Carfilzomib, Dexamethasone, Nivolumab	IV	1
NCT02101944	Recruiting	Anemia, Hypercalcemia, Lytic Bone Lesion, Osteopenia, Pathologic Fracture, Recurrent Plasma Cell Myeloma, Refractory Plasma Cell Myeloma	Pelareorep, Carfilzomib, Dexamethasone	IV	1
NCT01166542	Completed	Carcinoma, Squamous Cell of the Head and Neck	REOLYSIN, Carboplatin, Paclitaxel, Placebo	IV	3
NCT00984464	Completed	Metastatic Melanoma	REOLYSIN, Carboplatin, Paclitaxel	IV	2
NCT00753038	Completed	Carcinoma, Squamous Cell of the Head and Neck	REOLYSIN, Carboplatin, Paclitaxel	IV	2
NCT00503295	Completed	Osteosarcoma, Ewing Sarcoma Family Tumors, Malignant Fibrous Histiocytoma, Sarcoma, Synovial, Fibrosarcoma, Leiomyosarcoma	REOLYSIN	IV	2
NCT00998192	Completed	Metastatic or Recurrent Squamous Cell Carcinoma of the Lung	REOLYSIN, Paclitaxel, Carboplatin	IV	2
NCT00528684	Completed	Malignant Glioma	REOLYSIN	IV	1
NCT02723838	Withdrawn	Muscle-invasive Transitional Cell Carcinoma of the Bladder	REOLYSIN, Gemcitabine, Cisplatin	IV	1
NCT02620423	Completed	Pancreatic Adenocarcinoma	REOLYSIN, Chemotherapy, Gemcitabine, Irinotecan, Leucovorin, 5-fluorouracil, Pembrolizumab	IV	1

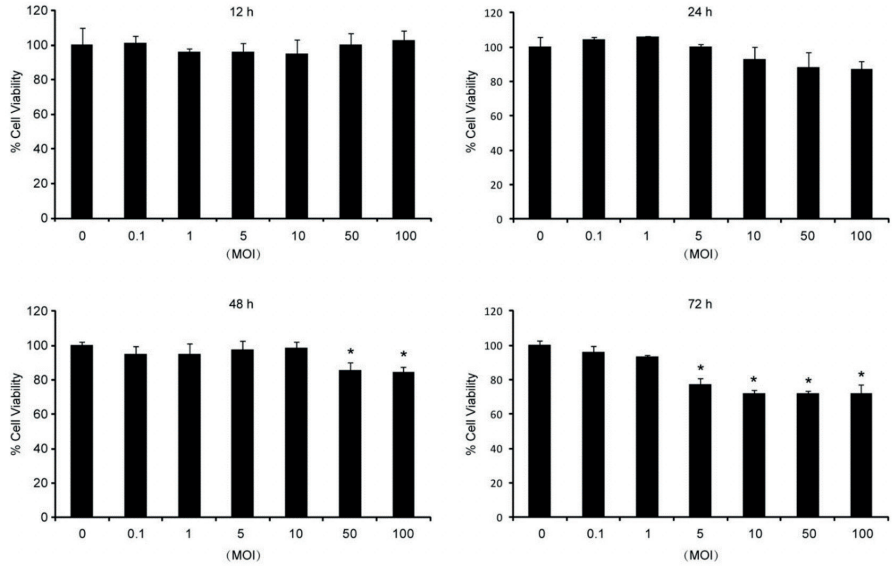

Figure 1. The viability of hUC-MSCs infected with reoviruses at different MOIs (0, 0.1, 1, 5, 10, 50, and 100) at 12, 24, 48, and 72 h. The cells were incubated for 2 h at 37°C before the culture medium was replaced. After incubation at the indicated times (12, 24, 48, and 72 h), the CCK8 assay was employed to assess cell viability. Data are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. $^{*}p < 0.05$ *vs.* non-infected hUC-MSCs (MOI = 0).

Coulter, Inc., Brea, CA, USA) by expression of the following cell-surface markers: cluster of differentiation (CD) CD44, CD73, CD90, and NOT-hUC-MSCs. The hUC-MSCs were seeded into T25 flasks at 10^6 cells/flask. The next day, hUC-MSCs were exposed to reovirus (MOI of 1) for 2 h and cultured in a growth medium for 72 h. After staining with antibodies in accordance with the manufacturer's instructions, the cells were washed twice with stain buffer to remove unbound antibodies, resuspended in 500 µL of stain buffer, and then subjected to flow cytometry.

Adipogenic and Osteogenic Differentiation Assays

The hUC-MSCs were inoculated into the wells of 6-well plates at 10^4 cells/well and then infected the next day reoviruses at a MOI of 1. After 72 h of culture, adipogenic and osteogenic differentiation was initiated as described previously[20,21]. For adipogenic differentiation, the cells were exposed to adipogenesis induction medium (DMEM containing 10% FBS), 10 µg/mL of insulin, 1 µM dexamethasone, 0.5 mM methyl isobutyl xanthine, and 100 µM indomethacin (all from Sigma-Aldrich Corporation, St. Louis, MO, USA). After 3 days of induction, the medium was replaced with adipogenesis maintenance medium (DMEM, 10% FBS and 10 µg/mL insulin) for 1 day. Two different media (induction and maintenance) were used alternately three times. At 12 days after initiation of differentiation, the cells were fixed and stained with Oil-Red-O (Sigma-Aldrich Corporation, St. Louis, MO, USA) for lipid droplets, which were photographed using a conventional inverted microscope (Olympus Corporation, Tokyo, Japan) under ×100 magnification.

Differentiation was induced by culturing cells in osteogenic differentiation medium (DMEM supplemented with 10% FBS, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 µM ascorbic acid 2-phosphate; all from Sigma-Aldrich Corporation, St. Louis, MO, USA). The medium was refreshed every 3 days for 21 days. Osteogenesis was confirmed by staining to highlight calcification of the extracellular matrix. Staining with Oil Red O and Alizarin Red S was quantified by measuring absorbance at 540 nm.

Wound-Healing Assays

Three days before infection with reovirus at a MOI of 1, equal numbers of hUC-MSCs (10^6) were seeded into 60-mm^2 cell culture plates. A scratch was created by scraping the cell monolayer in a line with a 200-µL sterile pipette tip. Cells were washed with phosphate-buffered saline (PBS; Gibco, Gaithersburg, MD, USA) to remove debris and the culture medium was replaced to

allow migration for 12 h. Cell migration images were obtained when scrape wounds were introduced (0 h) and at 12 h after wounding using an Olympus IX83 microscope (Olympus, Tokyo, Japan). Wound closure was measured using ImageJ software (https://imagej.nih.gov/ij/). All experiments were repeated at least three times.

Electrical Cell Substrate Impedance Sensing Analysis (ECIS) Analysis

ECIS analysis was conducted to assess cell proliferation and migration. Before infection with reovirus at a MOI of 1, equal numbers of hUC-MSCs (10^4 cells/well) were seeded into an electrode (Applied Biophysics, Inc., Troy, NY, USA). After the cell impedance curve was stable, the electric shock mode was turned on. Cell impedance continued to be monitored after washing with PBS to remove debris and replacing the culture medium. Changes in impedance were analyzed using Applied BioPhysics software.

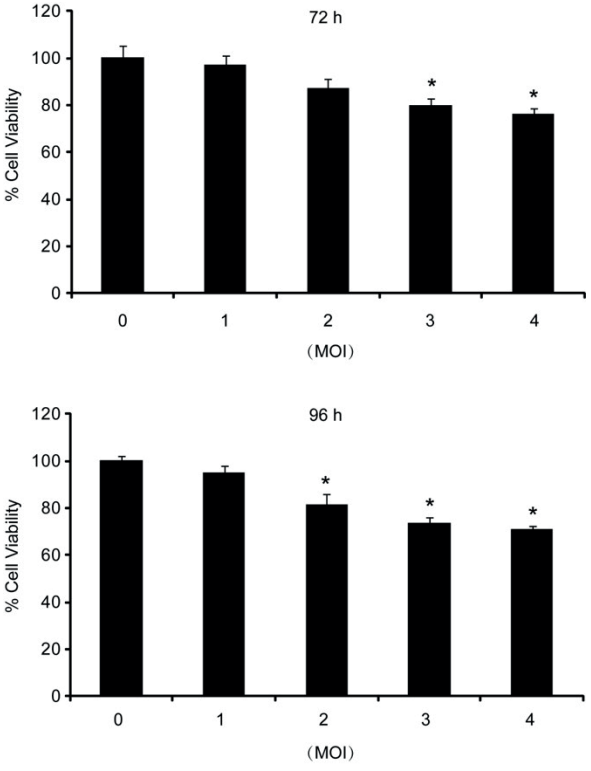

Figure 2. Effect of reoviruses on the viability of hUC-MSCs. hUC-MSCs were infected with reoviruses at different MOIs (0, 1, 2, 3, and 4) and incubated for 2 h at 37°C before the cell culture medium was replaced. After incubation at the indicated times (72 and 96 h), the CCK8 assay was performed to assess cell viability. Data are presented as the mean and SEM of at least three independent experiments. $^*p < 0.05$ *vs.* non-infected hUC-MSCs (MOI = 0).

Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 19.0. (IBM Corporation, Armonk, NY, USA). One-way analysis of variance (ANOVA) was used to assess differences between groups. Pearson's correlation analysis was used to assess correlations. A two-sided probability (p) value of < 0.05 was considered statistically significant.

Results

Reovirus Infection Had no Effect on the Viability of hUC-MSCs

The CCK-8 assay was employed to assess the viability of hUC-MSCs infected with reovirus at different MOIs (0, 0.1, 1, 5, 10, 50, and 100) at 12, 24, 48, and 72 h. As shown in Figure 1, cell activity decreased significantly at a MOI of more than 5, while there was no significant change at a MOI of less than 1 within 96 h (Figure 2). These results indicated that reovirus at a MOI of 1 had no effect on the viability of hUC-MSCs.

Reovirus Infection Had no Effect on the Proliferation of hUC-MSCs

ECIS has been used for a variety of applications, such as measuring cell proliferation and toxicity, adhesion, cell barrier, migration, wound and repair, and cell infiltration[22-24]. Here, ECIS was used to assess cell proliferation. First, cells adhered to an electrode that formed impedance with the magnitude of impedance proportional to the coverage of the cell on the electrode. Changes in impedance were analyzed. Reovirus-infected hUC-MSCs at a MOI of 1 had the same impedance as a control group within 70 h (Figure 3). These results indicated that reovirus infection had no effect on the proliferation capabilities of hUC-MSCs.

Reovirus Infection had no Effect on the Phenotype of hUC-MSCs

To characterize hUC-MSCs, P3-isolated cells were detected by flow cytometry. CD44, CD73, and CD90, three putative markers of MSCs, were detected on cell surfaces (Figures 4 and 5). The isolated cells did not express the monocyte markers CD31, CD11b, and HLA-DR, or the hematopoietic markers CD34 and CD45, in accordance with previous results[25]. After 72 h of reovirus infection, there was no significant difference in

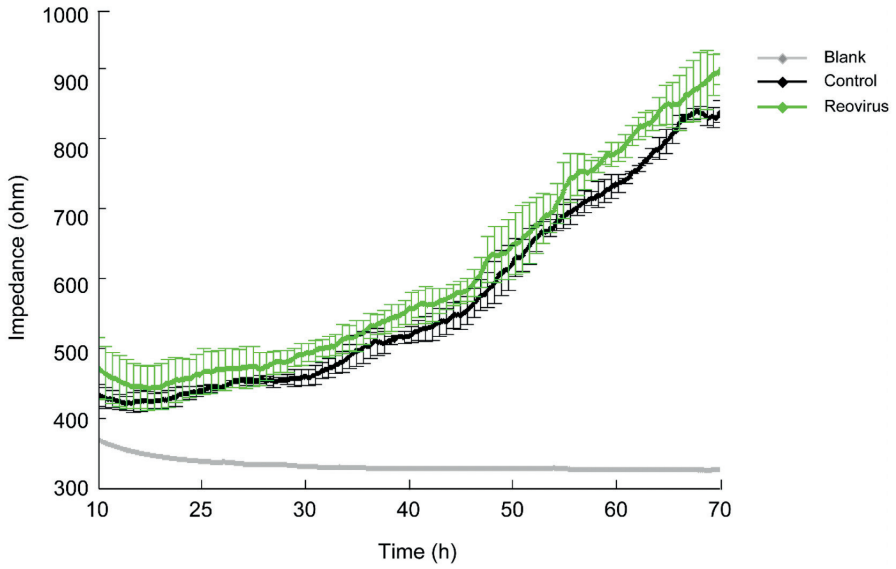

Figure 3. The ECIS assay was performed to assess cell proliferation. The hUC-MSCs (10^4 cells/well) were seeded into an electrode and infected with reovirus at a MOI of 1. Impedance was monitored using Applied BioPhysics software. The blank is cell culture medium only and the control is hUC-MSCs infected with reoviruses at a MOI of 0.

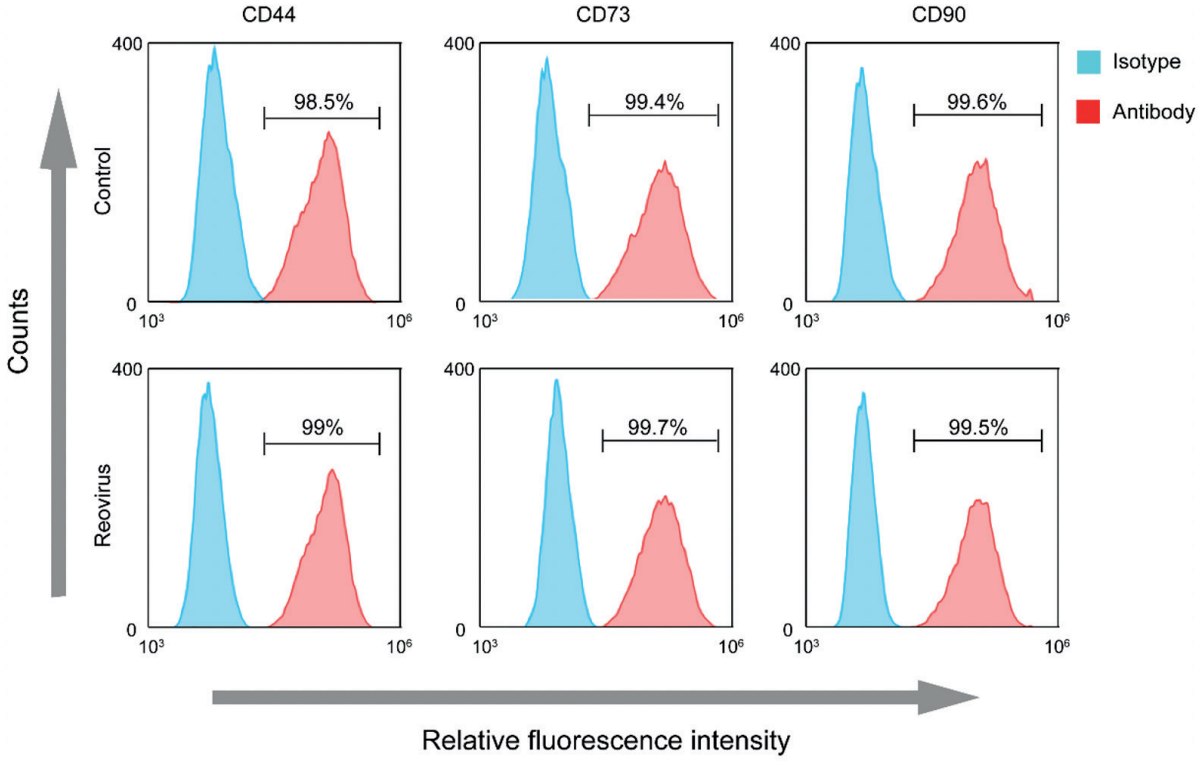

Figure 4. Expression levels of CD44, CD73, and CD90 of hUC-MSCs detected by flow cytometry at 3 days after reovirus infection at a MOI of 1.

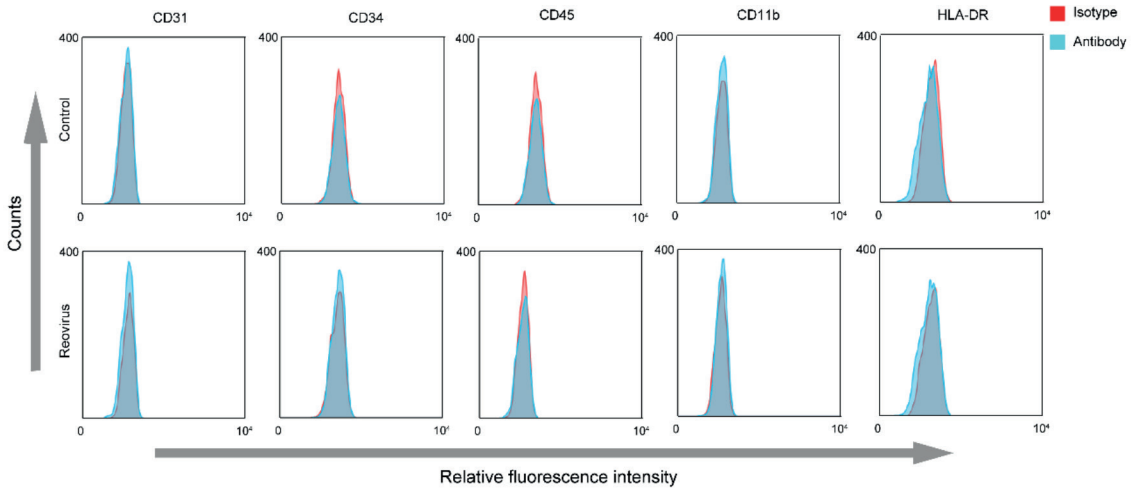

Figure 5. Expression levels of CD31, CD34, CD45, CD11b, and HLA-DR of hUC-MSCs detected by flow cytometry at 3 days after reovirus infection at a MOI of 1.

marker expression on the cell surfaces as compared with the control group (Figures 4 and 5). These results showed that reovirus infection of hUC-MSCs had no effect on the expression of markers on the cell surface.

Reovirus Had no Effect on the Adipogenic and Osteogenic Differentiation Capacities of hUC-MSCs

Next, the adipogenic and osteogenic differentiation capacities of hUC-MSCs were compared in the presence *vs.* absence of reovirus. As shown in Figure 6A, the adipogenic and osteogenic differentiation efficiencies of hUC-MSCs were not diminished after reovirus challenge as compared with mock-treated hUC-MSCs. The results were confirmed by quantitative destaining (Figure 6B, C).

Reovirus Had no Effect on the Migratory Capacity of hUC-MSCs

To evaluate the migratory capacity of hUC-MSCs after reovirus infection, cells were inoculated into 60-mm petri dishes and scratched with a 200-μL pipette tip in the presence or absence of reoviruses. Wound healing assays were initiated after 72 h of mock infection with reovirus at a MOI of 1. Cell migration was observed under a phase-contrast microscope after 12 h. As shown in Figure 7, there was no significant difference in wound healing in response to reovirus infection. Cell migration can be detected by ECIS[24,26]. Impedance values did not change in the presence or absence of reovirus (Figure 8). These results indicated that reovirus infection had no effect on the migratory capacity of hUC-MSCs.

Discussion

There have been many studies of oncolytic reovirus-based anti-cancer therapeutics[27]. To date, more than 30 clinical trials have been conducted with reoviruses, most of which administered the reoviruses intravenously. However, the efficacy of reoviruses is greatly reduced by intravenous administration due to neutralizing antibodies in adults. Therefore, virus-shielding strategies to improve viral transfer to tumors have been explored with the use of liposomes, nanoparticles, and cells[28]. The success of this strategy is dependent on four factors: effective *in vitro* virus loading, intracellular virus amplification, effective cellular targeting of tumor sites after systemic administration, and targeting of the virus to the tumor site[29].

Several studies have concluded that MSCs infected with herpes simplex virus can be used as delivery vehicles to target tumors in mice[30]. The survival of the mice was significantly prolonged as compared to the control groups. Other studies[29,31] have explored the delivery of oncolytic viruses, such as adenovirus and measles virus, to tumor-bearing mice. In the present research, hUC-MSCs were used as reovirus delivery vehi-

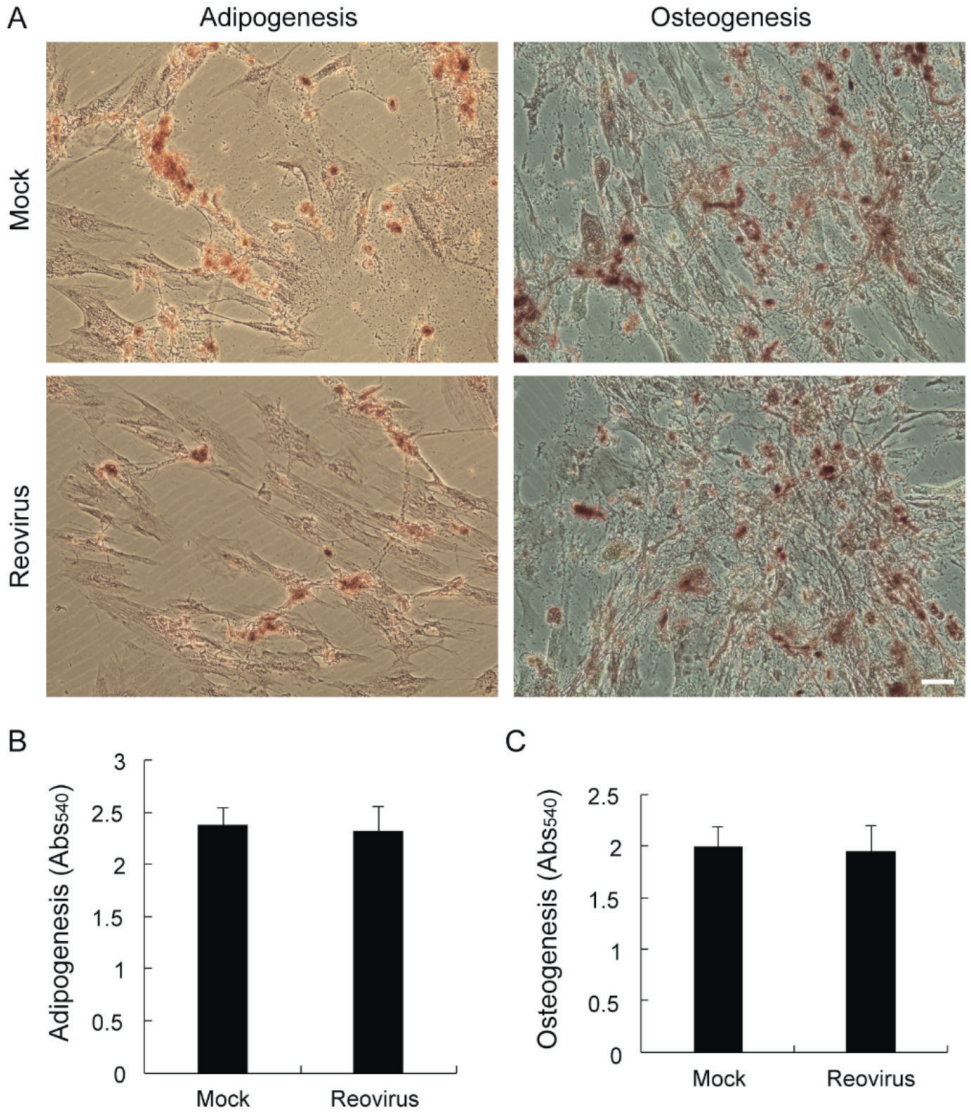

Figure 6. The adipogenic and osteogenic differentiation capacities of hUC-MSCs in the presence *vs.* absence of reoviruses (magnification × 100). hUC-MSCs were inoculated into the wells of 6-well plates at 10^4 cells/well. The next day, the cells were infected with reoviruses at a MOI of 1. After 72 h, the adipogenic and osteogenic differentiation capacities were assayed. The hUC-MSCs were stained with Oil Red O after 12 days of adipogenic differentiation (**A**, left) or Alizarin Red after 22 days osteogenic differentiation (**A** right). Bar = 100 μm. Quantitative data are shown (**B** and **C**). Values are presented as the mean ± standard deviation of three independent experiments.

cles for a number of reasons. First, hUC-MSCs have a stable genetic background and are easy to obtain with no harm to the donor. Second, hUC-MSCs are non-hematopoietic progenitor cells with strong self-renewal capability. Thus, the use of hUC-MSCs as delivery vehicles in preclinical research ensures the activities of both cells and viruses. Third, human umbilical cords are medical waste material, thus avoiding ethical disputes. Finally, based on preclinical studies investigating the potential of hUC-MSCs to deliver oncolytic viruses to tumor cells[15,32], we suggested that hUC-MSCs could be loaded with oncolytic reoviruses with minimal toxicity.

The biological activities of hUC-MSCs carrying reoviruses were also examined. The results of the CCK8 assay demonstrated that reovirus at low titers had little effect on the viability of hUC-MSCs and at a MOI of 1, had no effect on cell viability. Subsequently, the results of the ECIS assay indicated that reovirus challenge had no effect on the pheno-

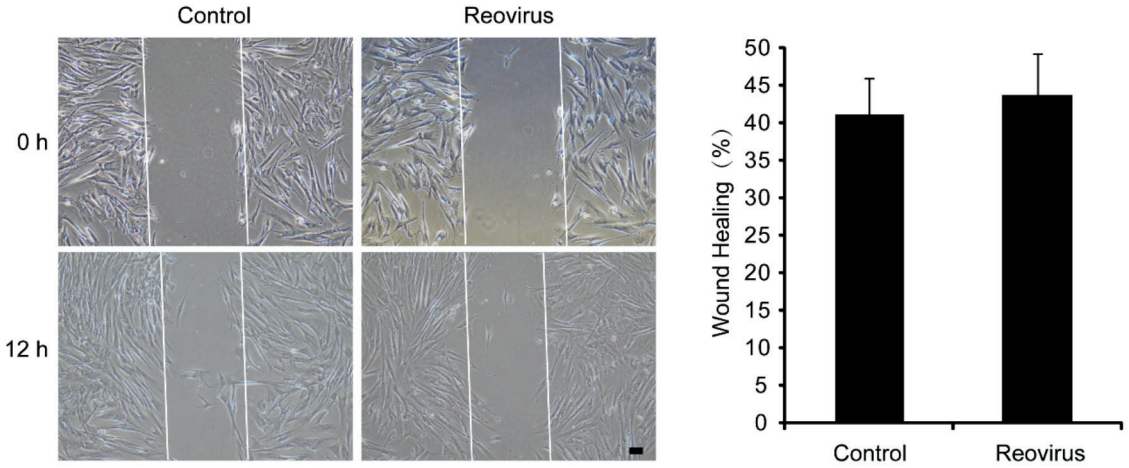

Figure 7. Cell migration as measured with the wound-healing assay (magnification × 100). At 3 days after infection of hUC-MSCs with reoviruses at a MOI of 1, a scratch was created by scraping the cell monolayer and images were obtained at 0 and 12 h after wounding using an Olympus microscope. Wound closure was assessed using ImageJ software.

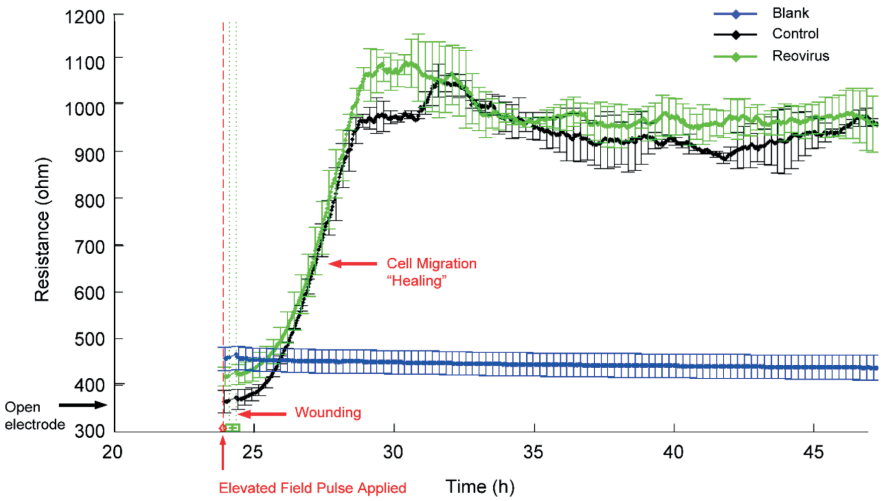

Figure 8. Assessment of cell migration using the ECIS assay. The hUC-MSCs infected with reoviruses (MOI = 1) were inoculated onto plates containing an electrode. At 72 h after infection, the electrode was turned on and cell debris was removed. Cell impedance was monitored using Applied BioPhysics software.

type or the differentiation and proliferation capacities of hUC-MSCs. Tumor tropism of virus delivery vehicles plays a crucial role in the anti-tumor efficacy of cell-based oncolytic virotherapies[33,34]. The results of the wound-healing and ECIS assays showed that reovirus infection did not impair the chemotactic migration of hUC-MSCs. Thus, the use of hUC-MSCs loaded with oncolytic reovirus may be considered as a novel strategy for virotherapy.

Although emerging evidence has demonstrated that virus-loaded MSCs induce a better anti-tumor effect than the administration of virus alone[34,35], there is still some controversy about the application of MSCs in anti-tumor therapy, as it is well-known that MSCs can promote tumor growth and metastasis by suppressing the immune response in the tumor microenvironment and other mechanisms[36-38]. Hence, the main focus of this investigation was the biological activities of hUC-MSCs after

reovirus infection. Nonetheless, future studies are needed to assess the therapeutic efficacy of reovirus-loaded MSCs and to elucidate the mechanism underlying the anti-tumor immune response in virotherapy.

Conclusions

The use of reoviruses had no effect on the biological activities (i.e., proliferation, marker expression, multipotency, and migration) of hUC-MSCs, which supports the potential of hUC-MSCs as effective cell carriers for clinical treatment with oncolytic anti-cancer reoviruses.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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