Comparison of the Effect of Adipose Mesenchymal Stem Cells-derived Secretome with and without Reovirus in CT26 Cells

Rezazadeh, A¹, Soleimanjahi H¹*, Soudi, S², Habibian, A¹

1. Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Department of Immunology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

*Corresponding Author: soleim_h@modares.ac.ir

ABSTRACT

Colorectal cancer is the fourth leading cause-related death of cancer which has significantly increased over the past three decades. New therapeutic approaches such as oncolytic viruses become very imperative recently to destruction of cancer cells. Use of mesenchymal stem cells secretome which are produced in response to variant conditions involves different paracrine molecules secretion that they have therapeutic potential in several chronic diseases. Mesenchymal stem cells and their derivative are used as a regenerative medicine, but there is ambiguity in the function of these cells in control of malignancy. In this study, we aim to examine the apoptotic effect of secretome derived from mesenchymal stem cells affected by encompassing oncolytic reoviruses. Mesenchymal stem cells were cultured after separation from abdominal adipose tissue of BALB/c mice. After three passages, the cells infected by reovirus at Multiplicity of Infection (MOI) of 1 Plaque-Forming Unit (PFU) per cell. Uninfected and infected secretome with reovirus were collected separately. The colorectal cancer CT26 cells were confronted with uninfected secretome, infected secretions, reovirus as positive control, and Dulbecco's Modified Eagle Medium/High Glucose (DMEM/HG) as negative control separately. Finally, apoptosis and necrosis were evaluated by flow cytometry. The infected secretome with reovirus is capable to induce apoptosis
more than uninfected secretome in CT26. However, supernatant of reovirus infected cells is more capable than others to induce cell death in comparison with infected secretome. Infected mesenchymal stem cells with oncolytic reovirus produce a type of condition media that enhance apoptosis induction and could have therapeutic effect on cancer cells. However, tumoral cells which confronted with the oncolytic reovirus showed more capability in inducing apoptosis in CT26 cells. As a result, the use of oncolytic virus and infected secretome are more effective than uninfected secretome in inducing apoptosis.

**Keywords:** Oncolytic Reovirus, Secretome, Colorectal Cancer Cell, Apoptosis, Cancer Therapy

**INTRODUCTION**

Cancer is a group of disease in which the abnormal cell starts autonomous growth and unlimited proliferation (1, 2). In colorectal cancer, mutations in Kirsten rat sarcoma viral oncogene homolog (KRAS) are very common (1). Using the new approaches such as virotherapy, oncolytic viruses and targeting tumor cells destruct cancer cells directly and stimulate the immune system which acts against tumor to omit it. Oncolytic viruses do not have the ability to enter and replicate in every cells. Two main factors in choosing their target cells are the presence of surface markers for binding to the target cells and permissiveness of target cells to replicate (3). Oncolytic viruses have the ability to enter the cancerous and normal cells around the tumor, but cancer cells have been weakened in antiviral mechanisms (4). When cell encounter to the virus, especially dsRNA virus such as reovirus, it is prevented from the proliferation of virus by activating the Protein Kinase R (PKR) pathway, but in cancer cells such as colorectal cancer, the Rat sarcoma viral oncogene homolog (RAS) pathway is active and it can block the PKR pathway (5, 6). Homozygous
mutation in kras increases the expression of proliferative markers through MAPK (mitogen activated protein kinases) and PI3K (phosphoinositide 3 kinase) in CT26 cells (7). In infectious viral diseases, interferon plays inhibitor role in the innate immunity in which PKR will be active. During cancer cell proliferation, Ras prevents PKR and consequently reovirus can replicate easily in CT26 (8). Reoviruses activate NF-kB transcription factor and increase proapoptotic genes such as caspase-1, p53, fasL and death receptors such as DR4 (TRAILR1), DR5 (TRAILR2), TNF related apoptosis – inducing ligand (TRAIL) as mediators of apoptosis (9, 10). Reovirus is the member of the Reoviridae family that has double–stranded segments of RNA. There are three serotypes of this virus. Type 3 (T3D) has greater potency as oncolytic virus and is applied for clinical and pre-clinical studies. This virus activity is highly dependent on the RAS signaling pathway (11). The investigation was found the activation of the RAS pathway concomitant with reovirus replication cycle, lead to the induction of death through the pathways of apoptosis, necrosis and autophagy (12, 13). After the proliferation of oncolytic reovirus in cancer cells, apoptosis may cause as the main result of cell death (14).

Mesenchymal stem cells (MSCs) are used in new ways for the treatment of chronic diseases. The unique ability of these cells to be multi-lineage differentiation potentials and self-renewal makes them capable of repairing and replacing damaged tissue at the injury site (14). Therapeutic potential, local and systemic effects of these cells essentially carry out through their cell secretions, which is called Secretome (15). They contain different factors such as soluble proteins, free nucleic acids, fat, extracellular vesicles, exosomes, etc. (15, 16). Secretome from various tissue cells is specific and in different physiological and pathological situation changes (15). MSCs produce proteins that inhibit apoptosis by reducing the expression of pre-apoptotic factors such as BAX factor and increased anti-apoptotic factors such as BCL2. This action of MSCs in health and cancer
conditions are different (17). Secretome-derived MSCs acts as a double edged sword for treating cancer. On one hand, can increase the proliferation of cancer cells, increase tumor growth, and inhibit apoptosis, on the other hand, increase the apoptosis in cancer cells (18).

In recent years, positive results have been reported on the ability of oncolytic viruses to destroy various types of cancer cells. For example, in 2017, Herpes Simplex Virus type 2 (HSV2) oncolytic viruses induced death in cells when exposed to CT26 (19). In another research, MSCs derived from bone marrow and adipose tissue infected with a specific dose of Newcastle disease virus, co-cultured with glioma cells and derivative secretome collected. The rate of cell death by the secretome of infected cells versus the oncolytic virus increased (20).

In this study, we compared the CT26 cell death as a colorectal cancer model after encompassing to infected and uninfected secretome. MSCs produce different secretion under various conditions. In a normal situation these cells inhibit apoptosis. The utilized MSCs in the current study do not have an experimental inflammatory condition or co-cultured with cancer cells. Thus, it may effect on secretion inhibitory characterization of lethal oncolytic activity and reduces its power. We hypothesize that the infected secretome has a greater effect on the induction of death than uninfected secretome, so contaminated secretome will be more powerful than not contaminated secretome to induce death in cancer cells.

MATERIALS AND METHODS

Isolation of mesenchymal stem cells from abdominal adipose tissue: MSCs were isolated from abdominal adipose tissue of male BALB/c mice aged between 6-8 weeks, obtained from Pasteur Institute of Iran. The collagenase I enzyme was used for tissue digestion. Following enzymatic digestion of adipose tissue, it was cultured in DMEM/HG (ATOCEL) containing 15% fetal bovine
serum (FBS) under a standard condition of temperature and pressure, 37°C and 5% CO2. The medium was changed every 3 days to cells reach to 70% confluency and preparation for subculture (6, 16).

**Phenotypic characterization of MSCs:** After three passages, MSCs were harvested and characterized by the antibody of a cluster of differentiation (CD) markers (Biolegened, USA): FITC-CD45 as a negative signal and PE-CD29, PE-CD90 as positive signals with flow cytometry assay (FAScanto II) (14).

**Reovirus propagation titer:** To increase the titer of reovirus, L-929 cells were used as the more susceptible host for proliferation. Following infecting the cells with Reovirus particles (MOI:1) supernatant was collected after presence of cytopathic effects (CPE). This cycle was carried out consecutively and the virus titration was determined by 50% Tissue Culture Infectious Dose (TCID50) assay. For this purpose, a serial dilution of the viruses has been prepared and added to each column of 96-well plates of L929 cell culture with 80% confluency. After 48 hours, each well was checked for CPE and virus titration was calculated with the Reed-Muench method (21).

**Preparation of MSCs condition media and infected condition medium:** Since various proteins are a major part of the condition media, proteins of FBS should be eliminated or reduced during last round of mesenchymal stem cells propagation to collect purified condition media. For this purpose the serum level was decreased by increasing the passage numbers. By removing the serum at the confluency of 70% MSCs in the third passage, condition media was collected after 48 hours. On the other hand, at the end of the third passage MSCs were infected by reovirus (MOI:1). Infected condition media was collected after 48 hours before infected MSCs destroyed completely.
Then simple conditioned media and infected condition media were centrifuged at 4000 Revolutions per minute (rpm) for 30 minutes and filtered by 0.22 micrometer (µm) filter separately to use for the following steps (21).

**Titer of virus in infected secretome:** In the third passage, MSCs were infected by $10^7$ titration of reoviruses (MOI equal to one). After collecting the infected secretome, the amount of virus that released in the secretome was measured by the TCID50 assay in the same way which described previously (21).

**Bradford protein assay:** It is a quick analytical technique that used to measure the concentration of proteins in a solution. In this test, 20 microliter (µL) of uninfected and infected secretome at the third passages were mixed with Bradford solution (Coomassie Brilliant Blue G-150, Ethanol and citric acid) then absorbance was determined by ELISA microplate reader at 540 nanometer (nm). The amounts of secretory proteins of stem cells were determined in infected an uninfected conditioned media to compare between their protein contents (22).

**Flow cytometry to measure Apoptosis and Necrosis:** CT26 cells as a colorectal cancer cell line and L929 cells as a control cell were cultured in 12-well plates and confronted with uninfected secretome, infected secretome, reovirus (control positive) and DMEM/HG (control negative). CT26 and L929 cell lines were incubated till 72 hours to evaluate apoptosis and necrosis in each well by using Annexin-PI technique (Invitrogen) by flow cytometry (23).

**RESULTS**

**Immunophenotyping:** The obtained data from flow cytometry for negative and positive CD markers revealed that adipose-MSC in the third passage were positive for PE-CD29 (98%), PE-CD90 (70%) and negative for FITC-CD45 (>2%). (Figure 1)
**Reovirus propagation titer:** CPE was checked and results in each well calculated with Reed-Muech formula. It was indicated $10^7$ pfu/mL available virus.

**Bradford protein assay:** Formula $Y = 0.0028X - 0.036$ (X: amounts of samples proteins µg/ml, Y: OD blank-OD sample) was used to calculate the amount of uninfected secretome and infected secretome proteins (µg/ml) obtained from the standard chart based on the bovine serum albumin (BSA) concentration in the serial dilution of 0-500. As the results were shown in (Figure 2), the amount of protein in the uninfected and infected secretome at the third passage from 24 to 48 hours was increased but the level of proteins was decreased in infected secretome in comparison with uninfected ones.

**Titer of virus in infected secretome:** The titer of virus in infected secretome was $10^{3.5}$ pfu/mL after 48 hours in the third passage. This result indicated a high decrease in virus titer. MSCs were infected with $10^7$ pfu/mL, but significant reduction was observed in MSCs infected secretome.

**Flow cytometry to measure Apoptosis and Necrosis:** The cell death percentages of apoptosis and necrosis in CT26 cells were determined at 48 and 72 hours (Figure 3), and L929 cells at 72 hours (Figure 4) after encountering with uninfected, infected secretome, reovirus and DMEM/HG by using Annexin-PI technique (Invitrogen) by Flow Cytometry (Figure 5). The results of apoptosis in CT26 indicated that death caused by infected secretome was more than uninfected secretome and in reovirus was more than both and the rate of cell death have increased during 72 hours.
DISCUSSION

In this study, we evaluated the induction of death in colorectal cancer cell line by the oncolytic reovirus and its association with adipose-derived mesenchymal stem cells tissue secretion.

The results of flow cytometry showed that necrotic death in all samples was very low and the highest mortality was due to apoptosis. Necrosis is the normal response of the cell to physiological damage, whereas apoptosis is due to intracellular stimulation of death (24), so this is conceivable that the induced death was due to the activation of internal pathways in the CT26 and L929 cells. As can be seen in the results, induction of apoptosis is the most important pathway of cancer cell lysis by the reovirus that occurs through caspase-dependent pathways and activation of the RAS pathway. Reovirus induces apoptosis from both the internal and external pathways and by activating the upstream and downstream of Ras pathway, which contribute to proliferation and induction of innate, humoral and cellular immunity in the host (3, 25-27).

The results have shown that reovirus was more capable to induce death than infected secretome but comparing infected secretome and uninfected secretome, the infected secretome had the potential to induce more apoptosis. The clinical use of MSCs due to their high potential for proliferation and activation of various signaling pathways which are favorable for viruses (11, 14) but the results were showed a decrease in the titer of the virus in the infected secretome. The secretome contains micro-vesicles, nanovesicles and exosomes as vesicles, that are responsible for the packaging of cellular products. The decrease in virus titration in the infected secretome may be due to the virus packaging inside these vehicles and make them protected from the immune system. As the results showed, reoviruses and MSCs influence on each other's function. The association between them reduces the amount of protein secreted by the virus and the MSCs and decreases the virus's titer. Using adipose mesenchymal stem cells in treatment is like using a
double-edged sword. It can stimulate tumor growth and metastasis in cancer or can destroy tumor cells by inhibiting cell proliferation and inducing apoptosis (18). Although mesenchymal stem cells are capable of producing or assisting regeneration, this effect is strongly influenced by factors such as the source of mesenchymal stem cells, age and health of the donor, amount of serum consumed in the culture medium, passage number and pro-inflammatory environment. Mesenchymal stem cells which are isolated from a different source, have dissimilar biological impacts as a result of different transcripts. In principle, the ability of the MSCs to regulate the immune system depends on two factors: cell-to-cell communication and paracrine effects (20, 28) MSCs can regulate the cell cycle by enhancing the synthesis phase, thereby augmenting the proliferation of cancer cell, increasing tumor growth, inhibiting apoptosis, angiogenesis and metastasis in colorectal cancer. These procedures activate NF-κb pathway signal through the AMPK/mTOR pathway. Upon activation of the NF-κb pathway, level of interleukin 6 and 8, both play important roles in the development of colorectal cancer, were enhanced and lead to tumor growth (18). Contrary to this, Kazimirsky et al. used the Newcastle virus as an oncolytic virus. They encountered bone marrow-derived mesenchymal stem cells and adipose tissue with a specific dose of Newcastle virus and then co-cultured with glioma cells. The infected secretome compared with the same virus titers, induced a higher level of apoptosis in tumor cells. Their finding implies that various factors which secreted from infected secretome make glioma cells more susceptible to viral effects (20). After co-culturing with glioma cells, the infected secretome has a greater ability to destroy cancer cells. In our study, MSCs did not experience any inflammatory or cancer conditions prior to collecting the uninfected secretome and infected secretome. This indicates that MSCs in different conditions produce different products, and cell-to-cell interactions determine the kind of secretion of these cells, therefore, the MSCs act as double-edged swords.
The interaction between MSCs and oncolytic reovirus remains unclear. Therefore, further studies are needed to investigate the interactions between oncolytic viruses and MSCs to find the best conditions to increase induced apoptosis in cancer cells.

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ETHICAL CONSIDERATIONS

The present study was approved by the ethics committee of Tarbiat Modares University with approval code (IR.TMV.REC.1396.686).

CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS

H.S.: Supervisor of study, contributed to study conception and design. H.S., A.R.: Conceptualized and designed the experiments. A.R., A.H.: Carried out experiments and acquired the data. A.R., H.S., S.S.: Interpreted the data and carried out data analysis and statistical analysis. A.R., A.H.: Drafted the manuscript. A.R., H.S., S.S.: Wrote the manuscript. All authors read and approved the final manuscript.
REFERENCES

**Figure 1: Immunophenotyping:** The obtained data from flow cytometry for CD markers revealed that adipose-MSC in the third passage were positive for PE-CD29 (98%), PE-CD90 (70%) and negative for FITC-CD45 (>2%).
**Figure 2. Bradford protein assay results:** The amount of protein (µg/ml protein in 24, 36 and 48 hours after infected and uninfected secretome collecting), in the uninfected secretome and infected secretome at the third passage was increased but the level of proteins was decreased in infected secretome in comparison with uninfected ones.
**Figure 3. Apoptosis and Necrotic of CT26 after 48 and 72 hours:** death percentages of apoptosis and necrosis in CT26 cells were determined at 48 and 72 hours after encountering with uninfected secretome, infected secretome, reovirus and DMEM/HG by using Annexin-PI technique (Invitrogen) by Flow Cytometry.
Figure 4. Apoptosis and Necrotic of L929 at 72 hours: The death percentages of apoptosis and necrosis in L929 were determined at 72 hours after encountering with uninfected secretome, infected secretome, reovirus and DMEM/HG by using Annexin-PI technique (Invitrogen) by Flow Cytometry.
Figure 5: Flow cytometric results of apoptosis and necrosis.

1- CT26/48h/SEC, 2- CT26/48h/SEC, REO, 3- CT26/72h/SEC 4- CT26/72h/SEC, REO