Archives of Razi Institute, Vol. 77, No. 2 (2022) 615-622

DOI: 10.22092/ARI.2021.353845.1615

Original Article

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

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Received 12 March 2021; Accepted 23 May 2021
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Abstract

Colorectal cancer is the fourth leading cause of cancer-related deaths that has significantly increased over the past three decades. New therapeutic approaches, such as oncolytic viruses, have become very imperative recently to destroy cancer cells. The use of mesenchymal stem cells (MSCs) secretome that is produced in response to variant conditions involves different paracrine molecules secretion that has therapeutic potential in several chronic diseases. Mesenchymal stem cells and their derivatives are employed as regenerative medicine; nevertheless, there is ambiguity in the function of these cells in the control of malignancy. This study aimed to examine the apoptotic effect of secretomes derived from MSCs 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 were infected by reovirus at the multiplicity of infection of 1 plaque-forming unit per cell. Uninfected and infected secretomes with reovirus were collected separately. The colorectal cancer CT26 cells were confronted with uninfected secretome, infected secretions, reovirus as a positive control, and Dulbecco's Modified Eagle Medium/High Glucose as negative control separately. Finally, apoptosis and necrosis were evaluated by flow cytometry. The infected secretome with reovirus was capable to induce apoptosis more than the uninfected secretome in CT26. However, the supernatant of reovirus infected cells was more capable to induce cell death, in comparison to the infected secretome. Infected MSCs with oncolytic reovirus produced a type of condition media that enhanced apoptosis induction and could have a therapeutic effect on cancer cells. Nonetheless, tumoral cells 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

1. Introduction

Cancer is a group of diseases 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 the tumor to omit it. Oncolytic viruses lack the ability to enter and replicate in every cell. Two main factors in choosing their target cells are the presence of surface markers for binding to the target cells and the permissiveness of target cells to replicate (3). Oncolytic viruses have the ability to enter the cancerous and normal cells around the tumor; however, cancer cells have been weakened in antiviral mechanisms (4). When cells encounter the virus, especially double-stranded ribonucleic acid (RNA) virus (e.g., reovirus), the proliferation of virus is prevented by activating the protein kinase R (PKR) pathway; nevertheless, 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 mitogen-activated protein kinases and phosphoinositide 3 kinases in CT26 cells (7). In infectious viral diseases, interferon plays an 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 nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) transcription factor and increase proapoptotic genes, such as caspase-1, p53, and fas ligand, and death receptors (DRs), such as Death receptor 4 (DR4), also known as TRAIL receptor 1 (TRAILR1), Death receptor 5 (DR5), also known as TRAIL receptor 2 (TRAILR2), and tumor necrosis factor-related apoptosis, inducing ligand (TRAIL) as mediators of apoptosis (9, 10).

Reovirus is a member of the *Reoviridae* family that has double-stranded segments of RNA. There are three serotypes of this virus, among which, type 3 (i.e., T3D) has greater potency as an 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 has revealed the activation of the RAS pathway concomitant with the reovirus replication cycle has led 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 develop 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 tissues at the injury site (14). Therapeutic potential and local and systemic effects of these cells are essentially carried out through their cell secretions, which is called secretome (15). They contain different factors, such as soluble proteins, free nucleic acids, fat, extracellular vesicles, and exosomes (15, 16). Secretome from various tissue cells is specific and affected by different physiological and pathological situation changes (15). Mesenchymal stem cells produce proteins that inhibit apoptosis by reducing the expression of pro-apoptotic factors, such as BAX (BCL2 Associated X, Apoptosis Regulator) factor, and increased anti-apoptotic factors, such as B-cell lymphoma 2. This action of MSCs in health and cancer conditions is different (17). Secretome-derived MSCs act as a double-edged sword for curing cancer. On the one hand, it can increase the proliferation of cancer cells, boost tumor growth, and inhibit apoptosis; nevertheless, on the other hand, it increases 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. In 2017, herpes simplex virus type 2 oncolytic viruses induced death in cells when exposed to CT26 (19). In another research, derived MSCs from bone marrow and infected adipose tissue with a specific dose of Newcastle disease virus, co-cultured with glioma cells and derivative secretome, were collected. The rate of cell death by the secretome of infected cells versus the oncolytic virus increased (20).

In the current study, the CT26 cell death as a colorectal cancer model was compared after encompassing infected and uninfected secretome. Mesenchymal stem cells produce different secretions under various conditions. In a normal situation, these cells inhibit apoptosis. The utilized MSCs in the current study lacked an experimental inflammatory condition or were not co-cultured with cancer cells. Therefore, it might have affected the secretion inhibitory characterization of lethal oncolytic activity and reduced its power. We hypothesized that the infected secretome had a greater effect on the induction of death than the uninfected secretome; in this regard, contaminated secretome would be more powerful than uncontaminated secretome to induce death in cancer cells.

2. Materials and Methods

2.1. Isolation of Mesenchymal Stem Cells from Abdominal Adipose Tissue

Mesenchymal stem cells were isolated from abdominal adipose tissue of male BALB/c mice aged

between 6-8 weeks, obtained from Pasteur Institute of Iran, Tehran, Iran. The collagenase I enzyme was used for tissue digestion. Following enzymatic digestion of adipose tissue, it was cultured in Dulbecco's Modified Eagle Medium/High Glucose (DMEM/HG; ATOCEL, Budapest, Hungary) containing 15% fetal bovine serum (FBS) under a standard condition of temperature and pressure (i.e., 37°C and 5% CO_2). The medium was changed every 3 days to make cells reach 70% confluency and be prepared for sub-culture (6, 16).

2.2. Phenotypic Characterization of MSCs

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

2.3. Reovirus Propagation Titer

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

2.4. Preparation of MSCs Condition Media and Infected Condition Media

Since various proteins are a major part of the condition media, proteins of FBS should be eliminated or reduced during the last round of MSCs propagation to collect purified condition media. To this end, 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 h. Moreover, at the end of the third passage, MSCs were infected by reovirus (MOI:1). Infected condition media was collected after 48 h before the complete destruction of infected MSCs. Subsequently, simple conditioned media and infected condition media were centrifuged at 4,000 rpm for 30 min and filtered by a 0.22-µm filter separately to be used for the following steps (21).

2.5. Titer of Virus in Infected Secretome

In the third passage, MSCs were infected by10^7 titration of reoviruses (MOI equal to one). After collecting the infected secretome, the amount of released virus in the secretome was measured by the TCID50 assay according to what was described previously (21).

2.6. Bradford Protein Assay

It is a quick analytical technique that is applied to measure the concentration of proteins in a solution. In this test, 20 µL of uninfected and infected secretome at the third passages were mixed with Bradford solution (Coomassie Brilliant Blue G-150, Ethanol and citric acid); afterward, absorbance was determined by enzyme-linked immunosorbent assay microplate reader at 540 nm. The amounts of secretory proteins of stem cells were determined in infected and uninfected conditioned media to compare their protein contents with each other (22).

2.7. 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 for 72 h to evaluate apoptosis and necrosis in each well using the Annexin-PI technique (Invitrogen) by flow cytometry (23).

3. Results

3.1. Immunophenotyping

The obtained data from flow cytometry for negative and positive CD markers revealed that adipose-MSCs in the third

passage were positive for PE-CD29 (98%) and PE-CD90 (70%) and negative for FITC-CD45 (>2%) (Figure 1).

3.2. Reovirus Propagation Titer

Cytopathic effects were checked and the results in each well were calculated with the Reed-Muench formula. It indicated 10^7 pfu/mL available virus.

3.3. Bradford Protein Assay

Formula Y=0.0028 X-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 concentration in the serial dilution of 0-500. As is shown in figure 2, the amount of protein in the uninfected and infected secretome at the third passage from 24 to 48 h was increased; nonetheless, the level of proteins was decreased in the infected secretome, in comparison to uninfected ones.

3.4. Titer of Virus in Infected Secretome

The titer of virus in the infected secretome was $10^{3.5}$ pfu/mL after 48 h in the third passage. This result indicated a high decrease in virus titer. It was also revealed that MSCs were infected with 10^7pfu/mL; however, a significant reduction was observed in MSCs infected secretome.

3.5. Flow Cytometry to Measure Apoptosis and Necrosis

The cell death percentages of apoptosis and necrosis in CT26 cells were determined at 48 and 72 h (Figure 3), and L929 cells at 72 h (Figure 4) after encountering uninfected and infected secretome, reovirus, and DMEM/HG using Annexin-PI technique (Invitrogen) by Flow Cytometry (Figure 5). The results of apoptosis in CT26 indicated that more deaths were caused by infected secretome than uninfected secretome and reovirus caused even more deaths than both of these, and the rate of cell death increased during 72 h.

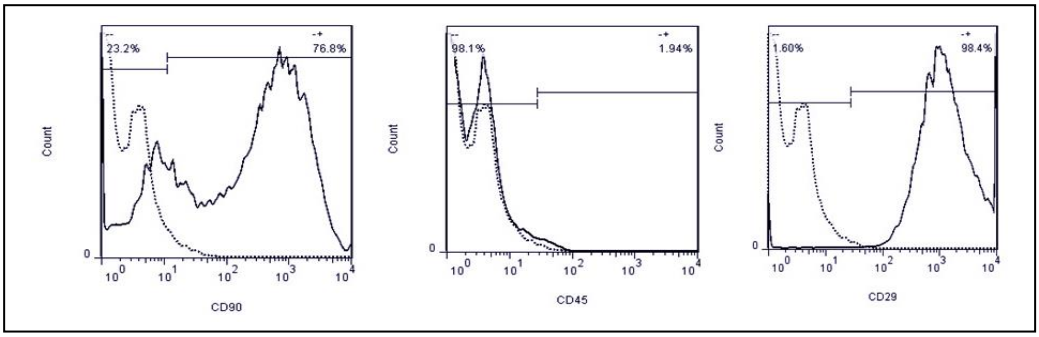

Figure 1. Immunophenotyping: The obtained data from flow cytometry for CD markers revealed that adipose-MSC in the third passage were positive for PE-CD29 (98%) and PE-CD90 (70%) and negative for FITC-CD45 (>2%).

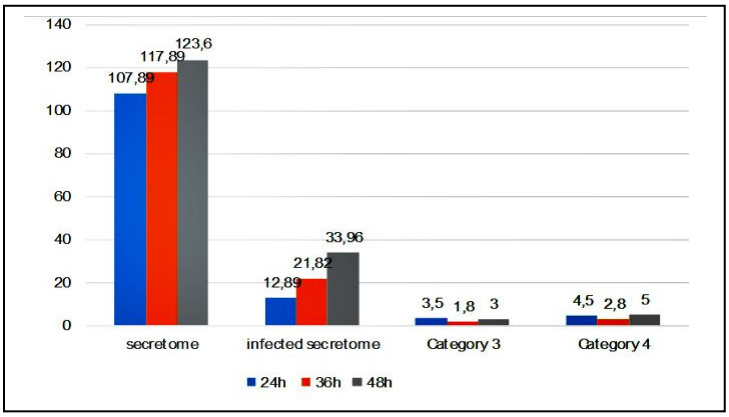

Figure 2. Bradford protein assay results: The amount of protein (µg/ml protein in 24, 36, and 48 h after collecting infected and uninfected secretome) in the uninfected secretome and infected secretome at the third passage was increased; however, the level of proteins decreased in the infected secretome, in comparison to the uninfected ones.

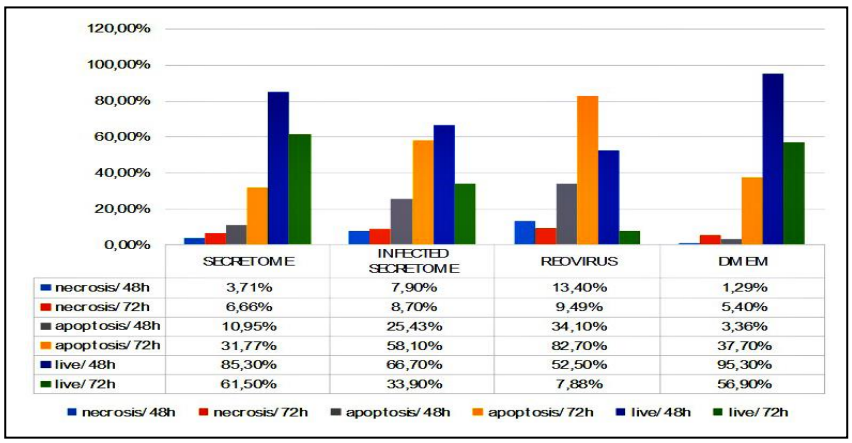

Figure 3. Apoptosis and necrotic of CT26 after 48 and 72 h: The death percentages of apoptosis and necrosis in CT26 cells were determined at 48 and 72 h after encountering uninfected secretome, infected secretome, reovirus, and DMEM/HG using Annexin-PI technique (Invitrogen) by Flow Cytometry

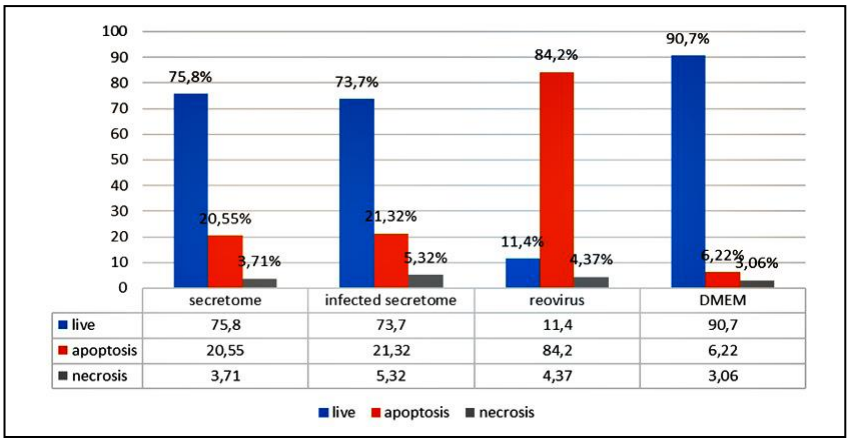

Figure 4. Apoptosis and necrotic of L929 at 72 h: The death percentages of apoptosis and necrosis in L929 were determined at 72 h after encountering uninfected secretome, infected secretome, reovirus, and DMEM/HG using Annexin-PI technique (Invitrogen) by Flow Cytometry

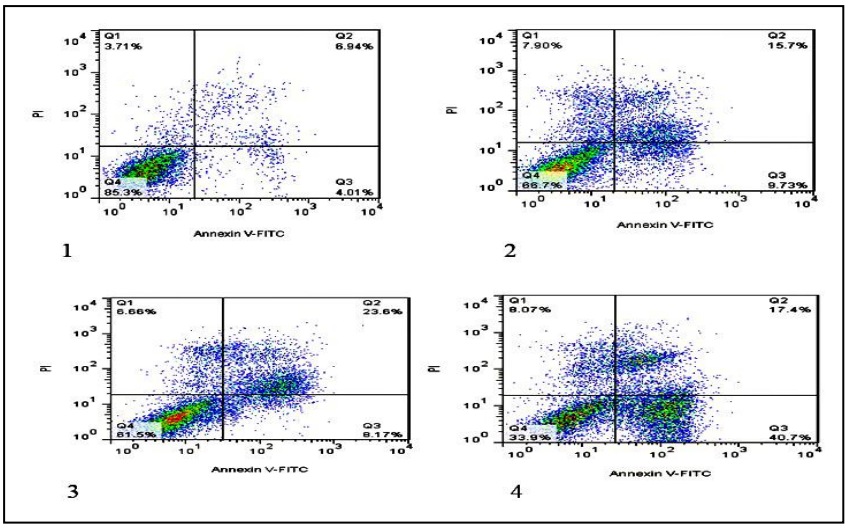

Figure 5. Flow cytometric results of apoptosis and necrosis
1- CT26/48h/SEC, 2- CT26/48h/SEC, REO, 3- CT26/72h/SEC 4- CT26/72h/SEC, REO

4. Discussion

This study evaluated the induction of death in colorectal cancer cell line by the oncolytic reovirus and its association with adipose-derived MSCs tissue secretion. The results of flow cytometry showed that necrotic death in all samples was highly 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); therefore, this is conceivable that the induced death was because of the activation of internal pathways in the CT26 and L929 cells. As can be seen in the results, the induction of apoptosis was the most important pathway of cancer cell lysis by the reovirus that occurred through caspase-dependent pathways and the activation of the RAS pathway. Reovirus induces apoptosis from both the internal and external pathways and by activating the upstream and downstream of the RAS pathway, which contributes to the proliferation and induction of innate, humoral, and cellular immunity in the host (3, 25-27).

The results showed that reovirus was more capable to induce death than infected secretome; however, the comparison of infected secretome and uninfected secretome revealed that the infected secretome had the potential to induce more apoptosis. The clinical use of MSCs is due to their high potential for proliferation and activation of various signaling pathways which are favorable for viruses (11, 14); nevertheless, the results indicated 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 that make them protected from the immune system. As the results have shown, reoviruses and MSCs influence each other's functions. The association between them reduces the amount of protein secreted by the virus and the MSCs and decreases the virus's titer. The use of adipose MSCs in treatment is a double-edged sword. It can stimulate tumor growth and metastasis in cancer or destroy tumor cells by inhibiting cell proliferation and inducing apoptosis (18). Although MSCs are capable of producing or assisting regeneration, this effect is strongly influenced by such factors as the source of MSCs, 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, namely cell-to-cell communication and paracrine effects (20, 28). Mesenchymal stem cells can regulate the cell cycle by enhancing the synthesis phase, thereby, augmenting the proliferation of cancer cells, increasing tumor growth, and inhibiting apoptosis, angiogenesis, and metastasis in colorectal cancer. These procedures activate the NF-kb pathway signal through the AMPK/mTOR pathway. Upon the activation of the NF-kb pathway, the level of interleukin 6 and 8, which both play important roles in the development of colorectal cancer, are 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 MSCs and adipose tissue with a specific dose of Newcastle virus, and subsequently, co-cultured with glioma cells. The infected secretome, compared to the same virus titers, induced a higher level of apoptosis in tumor cells. Their finding implies that various factors, which are secreted from infected secretomes, make glioma cells more susceptible to viral effects (20). The infected secretome has a greater ability to destroy cancer cells after being co-cultured with glioma cells. In our study, MSCs did not experience any inflammatory or cancer conditions prior to the collection of 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. In this respect, 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.

Authors' Contribution

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.

Ethics

The present study was approved by the Ethics Committee of Tarbiat Modares University with an approval code (IR.TMU.REC.1396.686).

Conflict of Interest

The authors declare that they have no conflict of interest.

Grant Support

The described results in this paper were part of a student thesis, which was supported by the grant (NO.75119) from the Research Deputy of Tarbiat Modares University, Faculty of Medical Sciences, Tehran, Iran.

Acknowledgment

This study was performed as an MSc thesis and financially supported by the Research Deputy of Tarbiat Modares University, Faculty of Medical Sciences, Tehran, Iran.

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