Mesenchymal stem cells promote caspase-3 expression of SH-SY5Y neuroblastoma cells via reducing telomerase activity and telomere length

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**ABSTRACT**

Objective(s): The use of mesenchymal stem cells in malignancies has attracted much attention due to their ability to deliver anticancer agents to tumors, including cytokines, chemokines, etc. This study aimed to investigate the effect of MSCs on the neuroblastoma SH-SYSY cells through proliferation/apoptosis, senescence assessment, telomere length, and telomerase activity in vitro. BAX and BCL2 were also examined as potential signaling pathways in this process.

Materials and Methods: For this reason, two cell populations (MSCs and SH-SYSY cells) were co-cultured on trans-well plates for 7 days. In a subsequent step, SH-SYSY cells were harvested from both control and experimental groups and subjected to flow cytometry, ELISA, real-time PCR, PCR-ELISA TRAP assay, and Western blotting assay for Ki67/Caspase3 investigation, β-Galactosidase assessment, telomere length, and telomerase activity assay. Also, expression of genes and proteins through real-time PCR and Western blotting demonstrated the involvement of the aforementioned signaling pathways in this process.

Results: It was found that MSCs contributed significantly to decrease and increase of Ki-67 and Caspase-3, respectively. Also, MSCs dramatically reduced the length of telomere and telomerase activity and increased the β-Galactosidase activity in a significant manner. In addition, significant increase and decrease were also seen in BAX and BCL2 gene and protein expressions, respectively.

Conclusion: These findings revealed that close interaction between MSCs and neuroblastoma cells causes inhibition of the SH-SYSY cell proliferation and promotes cell senescence via BAX and caspase-3 cascade pathways.

**Introduction**

Neuroblastoma is an extracranial tumor that causes death in childhood (1). Different therapeutic strategies include surgery, radiotherapy, chemotherapy, etc. But the outcome of these methods has definitely not been satisfactory. For this reason, over the past few years, researchers have increasingly focused on stem cell transplantation (2). Regarding the secretion of cytokines, chemokines, and anticancer factors, mesenchymal stem cells (MSCs) are ideally suited for cell-based therapy (3, 4). MSCs are multipotent cells isolated from various sources like amniotic fluid, bone marrow (BM), umbilical cord, adipose tissue and can differentiate into cells of mesodermal lineage (5, 6). MSCs are individually appropriate for the role of the carrier for anticancer therapies. In other words, MSCs are immunologically quiescent because of the low expression of major histocompatibility complex 1 (MHC1) and are able to migrate into the tumor site (7). Some studies indicated the capacity of MSCs homing to tumors that is favored in preclinical models of cancers (8, 9). In general, studies regarding the MSCs’ effects on cancer cells are contradictory. Such that, some studies reported inhibition effects of these cells on tumor growth and others indicated opposite effects (10, 11). MSCs may favor tumor proliferation via stimulation of the metastatic potential and also prevent tumor cell recognition by the immune system. Conversely, MSCs can exert anticancer activities by down-regulating activities through the expression of apoptotic molecules.

To date, the effects of MSCs on neuroblastoma growth and the mechanisms of influences on tumor progression via telomere length and telomerase activity are yet to be reported. Therefore, this study reveals the in vitro effects of MSCs on the proliferation and apoptosis of the SH-SYSY cells as neuroblastoma cell lines by investigating the Annexin-V and Caspase-3 assay, telomerase, and β-Galactosidase activity as well as telomere length. The possible involved signaling pathways like BAX as well as BCL2 were also investigated.

**Materials and Methods**

**Materials**

All materials related to cell culture as well as the culture polystyrene plates, if not otherwise specified, were obtained from SPL Life Sciences Co., Ltd. (Gyeonggi-

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Apoptosis induction of SH-SY5Y cells by MSCs

Cell culture
Adipose tissue-derived-MSCs and SH-SY5Y neuroblastoma cells were purchased from Royan Institute and Pasteur Institute of Iran, respectively. MSCs and SH-SY5Y cells were cultured in DMEM low glucose (Gibco, UK) containing 10% FBS (complete culture medium). The medium was replaced twice weekly during the cultivation of the cells. MSCs of passages 3–6 were used throughout the present study (12).

Characterization of MSCs
Purchased MSCs were characterized by flow cytometry with antibodies against cell surface markers CD73, CD44, CD31, and CD34. Preparation of cells for flow cytometry method was previously described by Fathi et al. (2020) (13). Briefly, nearly 10^6 MSCs were trypsinized and incubated with 10 μg/10^6 cells FITC-conjugated antibody CD31 and CD34 and PE-conjugated CD73, and CD44 in washing buffer (PBS supplemented with 3-5% FBS) on ice. In the end, the cells were washed with washing buffer, and the FACS instrument was used to quantify the fluorescence intensity of MSCs (14).

Co-culture of MSCs and neuroblastoma cell line (SH-SY5Y)
MSCs were seeded into 6-well trans-well plates at 10^5 cells/well in DMEM complete culture medium. After 24 hr of cells culture, 10^6 SH-SY5Y cells/well were added respectively into two groups: control group (culture of SH-SY5Y cells alone) and experimental group (co-cultured SH-SY5Y cells and MSCs). At the end of the co-culture period (7 days), two groups of cells were subjected to total RNA isolation for gene expression, Ki-67/caspase-3 assessment by flow cytometry, and protein extraction for Western blotting.

Flow cytometric investigation of apoptosis assay
To determine cell apoptosis in co-culturing SH-SY5Y cells with MSCs, Annexin-V staining was used. Annexin-V can bind to phosphatidylserine at the surfaces of apoptotic cells as a marker for apoptosis. Annexin-V expression was detected with antibodies against cell surface markers CD73, CD44, CD31, and CD34. Preparation of cells for flow cytometry with antibodies against cell surface markers 3-30, 3-44, and 3-12 was previously described by Fathi et al. (2020) (13). Briefly, nearly 10^6 MSCs were trypsinized and incubated with 10 μg/10^6 cells FITC-conjugated antibody CD31 and CD34 and PE-conjugated CD73, and CD44 in washing buffer (PBS supplemented with 3-5% FBS) on ice. In the end, the cells were washed with washing buffer, and the FACS instrument was used to quantify the fluorescence intensity of MSCs (14).

Real-time-PCR
After the end of treatment time (7 days), 10^6 SH-SY5Y cells/well from both groups of cells were collected, total RNA from the cells was isolated and cDNA was synthesized using a YTA kit (Yekta Tajhiz Azma, IRAN) (22). The mRNA expressions of target genes in this experiment included BAX, BCL2, and β-actin. The primer sequences used for real-time PCR are listed in Table 2.

Western blotting assay
Western blotting was used to determine whether measure apoptosis in 10^6 cells from both the control and experimental groups. accoring to manufacturer’s directions (Ref No: 11-8005-74, e-bioscience). FlowJo software ver. X.0.7 was then used to analyze the percentages of viable and apoptotic cells (15, 16).

Ki-67/caspase-3 investigation
Ki-67/caspase-3 assay was performed in control and experimental groups. In this regard, the cells were incubated with 0.2% Triton X-100. Next, the cells were stained with Ki-67 antibody for 30 min. Moreover, for the caspase-3 assay, cells were fixed using FCM fixation buffer and permeabiliyzed by FCM permeabilization buffer. Washed cells were stained using PE-conjugated anti-caspase. The flow cytometry data were analyzed with FlowJo software ver. X.0.7 (17).

Absolute telomere length (aTL) measurement and telomerase activity assay
For measuring the telomere length, the total DNA was isolated (18). The aTL measurement was done by real-time PCR as previously reported by Fathi et al. (2020) (19). Also, the primers used in this method were previously designed by O’Callaghan and Fenech (2011) (Table 1) (20). The telomerase activity of SH-SY5Y cells was measured by PCR-ELISA TRAP assay.

Senescence-associated β-galactosidase assay by ELISA
Cells (10^6 cells/wells) from both groups (control and experimental) were lysed and β-Galactosidase activity was assessed using the human β-Galactosidase enzyme-linked immunosorbent assay (ELISA) Kit (11539426001, Roche, UK) according to the manufacturer’s guidelines (21).

Western blotting assay
Western blotting was used to determine whether...
BAX/BCL2 signaling pathways are involved. For this examination, proteins were extracted from both control and experimental groups of SH-SY5Y cells and were electrophoresed using 12% polyacrylamide, and finally transferred onto PVDF membranes. Following that, the membrane was exposed overnight to primary antibodies for BAX and BCL2 (1:500, Santa Cruz Biotechnology, CA). Afterward, the membranes were incubated at 25 °C for 60 min with a secondary antibody (1:5000 Santa Cruz). Next, X-ray films were used to detect the presence of protein bands. Protein bands were quantified and represented as the ratio of target protein/β-actin and the values were then graphed (23, 24).

Statistical analysis

Values were considered statistically significant at \( P<0.05 \) using Graph Pad Prism version 6.01 using t-test and Two-way ANOVA followed by Dennett’s post hoc test.
co-culture period. In more detail, early apoptosis (Annexin+) was about 33.6% for SH-SY5Y cells exposed to MSCs, which was 2.52 times higher than that of the control group (13.3%). While it was only 4.11% for the cells in the late apoptotic stage (PI+) (Figure 2).

**Proliferation/apoptosis investigation by Ki-67/caspase-3 assay**

The effect of MSCs on cell proliferation of SH-SY5Y cells was investigated by Ki-67 expression (Figures 3A-E). The percentage of Ki-67 expression in the co-culture group (66.4%) was lower than that in the control group (82.4%) (Figures 3A-E) \( (P<0.05) \). Also, Figures 3F-J showed that caspase-3 levels in the co-culture group were increased about 3.56-fold compared with the control group \( (P<0.05) \).

**Investigation of αTL and telomerase activity**

As shown in Figure 4C, αTL was significantly decreased (14.60 Kbp) compared with the control group (57 Kbp) \( (P<0.01) \). Also, the telomerase activity was decreased by 68.5% in the experimental group compared with the control group (Figure 4D) \( (P<0.05) \).

**β-Galactosidase activity for cell senescence investigation**

As shown in Figure 5, the concentration of β-Galactosidase in the experimental group was 8.33 folds higher than that of the control group \( (P<0.05) \) (Figure 5).

**MSCs change mRNA and protein expression of BAX and BCL2 in the SH-SY5Y cell line**

As shown in Figures 6A and B, the protein expression
levels of BAX and BCL2 were significantly increased and decreased, respectively (P<0.05). In addition, the apoptotic BAX/BCL-2 ratio was increased by 7.7 folds in the co-cultured SH-SY5Y cell line (Figure 6C) (P<0.001). A significant increase and decrease in mRNA expression levels of BAX and BCL2, respectively, were also seen (Figure 6D) (P<0.05, P<0.01).

**Discussion**

In cancer treatment, stem cells can act as a promising delivery platform by homing to and delivering therapeutics into tumor foci (26). Studies regarding the role of MSCs in cancer cells are contradictory. Some of these are associated with their promotional effects, while others address their inhibitory effects (8, 27-28). The precise cellular and molecular mechanisms of MSCs' effects on cancers and tumor progression are yet to be elucidated. Our study presents evidence for the effects of MSCs on SH-SY5Y neuroblastoma cells by investigating Annexin-V and Caspase-3 assay, telomere length, telomerase, and β-Galactosidase activity assessment through BAX and BCL2 signaling pathways. To explore the hypothesis of this study, SH-SY5Y cells were co-cultivated with MSCs in a trans-well system for 7 days. Both the control and experimental groups of SH-SY5Y cells were collected at the end of co-culture time, and flow cytometry, real-time PCR, PCR-ELISA TRAP assay, Western blot, and ELISA were performed. Based on the results of this study, the experimental group (SH-SY5Y co-cultured with MSCs) exhibited a dramatic decline in telomere length and telomerase activity which was accompanied by a remarkable increment in the β-Galactosidase activity by more than 3.9, 1.45, and 8.33 fold, respectively, in comparison with the control group consisting of SH-SY5Y cells alone. Due to high telomerase activity in cancer, its inhibition is a great interest and strategy in cancer treatment. Despite numerous studies, the underlying molecular and cellular mechanisms have yet to be fully elucidated.

Previous investigations have reported that telomere length and telomerase activity were considered as useful diagnostic and prognostic markers in some neoplasms such as neuroblastoma (31). So that a high correlation was demonstrated between telomere length and malignancies in brain tumors including meningioma and glioblastomas (32). In one study (2004), it was shown that shorter and elongated telomeres were accompanied by favorable and poor prognoses, respectively (33). With these explanations, the results of our study regarding the telomere length are consistent with the previous findings. Based on the results of previous studies regarding the involved mechanisms in telomere length change and telomerase activity, it was theorized that shortening of telomere length and telomerase activity may be strongly influenced by MSCs-secreted cytokines released (34). In this regard, a number of factors may have a contributory role, including IL-6, IL-8, and TGF-β.

Aside from measuring telomere length, we assessed telomerase and β-Galactosidase activity, as well as Annexin-V staining, Ki-67/Caspase-3 assay, BAX, and BCL2 levels. Clear identification of the mechanism of cell damage/death caused by MSCs is essential for assessing the biological response to cell therapy. In accordance with the Annexin-V results, it was well-identified that the early apoptotic cells showed a significant increment, with 13.3 apoptotic cells in

**Figure 5.** β-GAL activity assay of SH-SY5Y cells and SH-SY5Y cells co-cultured with MSCs compared with the control group, the experimental group displayed a higher level of β-galactosidase activity. Values are mean ± SD from independent experiments (*P<0.05, n=3)

**Figure 6.** Effect of MSCs on apoptosis-related gene and protein expression. (A and B) BAX and BCL-2 protein expression (C) BAX/BCL2 ratio, (D) Relative level of mRNA expression of Bax and BCL2. Data are expressed as mean ± SD from three independent experiments (*P<0.05, **P<0.01, ***P<0.001, n=3)
the control group reaching 33.6% in the experiment group. Additionally, the late apoptotic cell level clearly increased from 2.35 to 4.11% after 7 days of co-culture.

Conclusion
We provided evidence that MSCs decreased telomerase activity and telomere length of SH-SY5Y cells, which was associated with apoptosis induction and an increase in the expression level of caspase-3. Furthermore, these effects result in increased and decreased expression of the BAX and BCL2, respectively, which are critical components of apoptotic signaling pathways. Finally, a cytokine antibody array method is suggested to investigate the mechanisms by which cytokines are secreted from MSCs, as well as the types of secreted cytokines and growth factors.

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Authors’ Contributions
EF as the main colleague contributed to performing experiments, data analysis, and manuscript writing; SV and SM as main colleagues were involved in manuscript writing; RF as the executive of the project was involved in conception and design, manuscript writing, and supervised the manuscript preparation.

Ethical Approval
Ethical consent was approved by an ethics committee at Tabriz University of Medical Sciences, Tabriz, Iran (Ethic Code No: IR.TBZMED.VCR.REC.1400.231).

Conflicts of Interest
The author(s) declare that there are no conflicts of interest.

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Apoptosis induction of SH-SY5Y cells by MSCs

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