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The dual inhibitory effect of adipose-derived mesenchymal stem cell secretome on JAK2/STAT3 and PI3k/AKT/mTOR signaling pathways

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Abstract

Stem cells are considered as promising candidates to effectively hinder the proliferation of different types of cancers including hepatocellular carcinoma, Kaposi's sarcoma, as well as gastric and breast cancers. Mesenchymal stem cells (MSCs) have attracted a lot of attention among the different categories of such cells. Adipose tissue offers unique advantages as a source of MSCs. Based on some studies, the released substances from MSCs which are regarded as secretome can modulate the growth and survival of tumor cells, along with immunity and angiogenesis by affecting different signaling pathways. JAK/STAT and PI3k/AKT/mTOR signaling pathways play the main role in regulating growth, proliferation, apoptosis, and cancer metastasis. This study aims to assess the cytotoxic effect of human adipose-derived MSC (adMSC) secretome on two cancerous cell lines by co-culturing each cancerous cell line with adMSCs and performing MTT assay, as well as evaluating simultaneous inhibitory effect of adMSC secretome on the expression rates of the genes related to JAK2/STAT3 and PI3k/AKT/mTOR signaling pathways by conducting real-time PCR after co-culturing. The results indicated that adMSC secretome did not exert cytotoxic effect against epidermoid carcinoma (A431) cell line, leading to increased cell viability. However, the percentage of viable cancerous cells significantly reduced following co-culturing of gastric adenocarcinoma (AGS) cell line with adMSCs, indicating different cytotoxic potency of adMSC secretome towards these cell lines. adMSC secretome downregulated the expression rates of Jak2, STAT3, PI3k, and mTOR genes in both co-cultured cell lines, despite different effects against A431 and AGS cell lines, indicating the significance of such signaling pathways in the growth and proliferation of each cancerous cell lines. The results provide opportunities for examining *in vitro* and *in vivo* cytotoxic potency of adMSC secretome against other types of cancers and further evaluation of its downstream mechanisms of action through cancer signaling pathways. Thus, they may lead to the use of adMSC secretome as a novel therapeutic agent in different types of cancers.

Keywords: Cancerous cell line, co-culture techniques, cytotoxicity, gene expression, mesenchymal stem cells, signaling pathways, secretome

1. Introduction

The incidence of cancer is increasing worldwide and the American Cancer Society reports that cancer continues to be the second most prevalent reason for mortality, following heart disease. In addition, cancer stands as the primary reason for death among women aged 40-79 and men aged 60-79 years old (Sung *et al.*, 2021). The survival rate may remain low due to a delay in cancer treatment, resulting in increasing advanced cases, despite the advancements in early detection and treatment strategies for various types of cancer over the past few decades (Siegel *et al.*, 2023). Therefore, innovative therapeutic strategies should be adopted to address advanced or metastatic cancers.

Stem cell therapy has become a hopeful strategy for addressing different forms of cancer including hepatocellular carcinoma, Kaposi's sarcoma, as well as gastric and breast cancers (Alzahrani *et al.*, 2018; Khakoo *et al.*, 2006; Li *et al.*, 2013; Liu *et al.*, 2020; Mohamed *et al.*, 2019; Pakravan *et al.*, 2017). Because of different source of origin, differentiation potency and the ability of transplant to variety of people, mesenchymal stem cells (MSCs) have attracted a lot of attention among the different categories of stem cells (Gopalarethinam *et al.*, 2023). They can be isolated from umbilical cord, amniotic membrane, adipose tissue, bone marrow, and other tissues (Bhat *et al.*, 2021; Cho *et al.*, 2019; Hendrijantini and Hartono, 2019; Zhang *et al.*, 2019). Adipose tissue, which offers unique advantages as a source of MSCs, can be easily obtained from the medical waste of bariatric

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surgery, resulting in eliminating any ethical concerns. Additionally, MSCs derived from adipose tissue (adMSCs) can be extracted from patients without the risk of immune rejection (Palencar *et al.*, 2019; Wyles *et al.*, 2015).

The MSCs can interact with tumor cells either by direct contact or by releasing certain substances such as chemokines, growth factors, cytokines, microvesicles, and exosomes with immunomodulatory effects (Crivelli *et al.*, 2017; Jayaramayya *et al.*, 2020). Based on some studies, these released substances, which are considered as secretome, inhibit cancer cell proliferation (Aslam *et al.*, 2021; Mirabdollahi *et al.*, 2019; Moeinabadi-Bidgoli *et al.*, 2022; Sousa *et al.*, 2023). The MSCs can affect different signaling pathways through their secretome (Chang *et al.*, 2022; Jantalika *et al.*, 2022; Ko *et al.*, 2023; Rezaei-Tazangi *et al.*, 2020; Sousa *et al.*, 2023; Yuan *et al.*, 2018) and induce cell apoptosis or suppress cell proliferation, migration, and invasion. Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) and phosphoinositide 3-kinase (PI3k)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathways play the main roles in controlling cell growth and proliferation, apoptosis, and metastasis in different types of cancers (Al-Husein *et al.*, 2020; Fattahi *et al.*, 2020; Liang *et al.*, 2020; Ma *et al.*, 2020; Mengie Ayele *et al.*, 2022; Miricescu *et al.*, 2020; Mohrherr *et al.*, 2020; Rah *et al.*, 2022; Tewari *et al.*, 2022; Wu *et al.*, 2017; Yang *et al.*, 2020). In addition, aberrant activation of PI3k/AKT/mTOR pathway may result in the development of resistance to apoptosis (Fattahi *et al.*, 2020; Tewari *et al.*, 2022). Based on some reports, simultaneous inhibition of JAK2/STAT5 and PI3k/AKT/mTOR signaling pathways was more effective in reducing cancer cell number, growth of tumor and metastasis, as well as increasing survival *in vivo* compared to only PI3k/AKT/mTOR pathway inhibition (Yeh *et al.*, 2013). Only PI3k/AKT/mTOR signaling pathway inhibition led to uncontrolled activation of the JAK/STAT one and occurrence of metastatic and aggressive behaviors in tumor cells.

Based on the results, (Yeh *et al.*, 2013), simultaneous inhibition of STAT3 and JAK2, as key molecules in JAK2/STAT3 signaling pathway, and PI3k and mTOR, as vital molecules in PI3k/AKT/mTOR signaling pathway, can be regarded as prospective therapeutic targets for cancer treatment.

This study seeks to evaluate the cytotoxicity of MSC-secretome on two distinct cancers including gastric adenocarcinoma (AGS, RRID: CVCL_0139) and epidermoid carcinoma cell lines (A431, RRID: CVCL_0037), as well as reviewing MSC-secretome effect on gene expression rates of Jak2, STAT3, PI3k, and mTOR for achieving a deeper comprehension of the molecular mechanisms by which MSCs exert their influence on cancerous cells.

2. Materials and methods

2.1. Cell culture

Gastric adenocarcinoma (AGS, RRID: CVCL_0139) and epidermoid carcinoma (A431, RRID: CVCL_0037) cell lines (purchased from Pasteur Institute of Iran) and mesenchymal stem cells derived from human adipose (adMSCs, IBRC: C11347, purchased from Iranian Biological Resource Center, Iran) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Bioidea, Iran) by adding 10% fetal bovine serum (FBS) (Bioidea) and incubated at 37°C in a humidified atmosphere, containing 5% CO_2. Every 48-72 h the culture medium was refreshed and passages 3-5 were utilized for further experiments.

2.2. Assessing cytotoxic effect of adMSC-secretome against cancerous cell lines

In order to determine the cytotoxic effect of adMSC-secretome against cancerous cell lines (A431 and AGS), each cancerous cell line was co-cultured with ad-MSCs for 96 h, followed by conducting MTT assay (Mohammadalizadeh *et al.*, 2022). Briefly, cell line (either A431 or AGS) was seeded in the lower compartments of each well in an insert-containing 6-well plate (SPL, China) at 1.5×10^5 cells/well in complete DMEM medium. Then, ad-MSCs were seeded in the upper compartments (the inserts with the pore size of 8µm) at a density of 6×10^4 cells/well and further incubated for 96 h in the same condition after being incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. Cancerous cell line was included as control without being co-cultured with ad-MSCs. In the next step, all of the inserts were taken out and cancerous cells were washed with phosphate buffered saline (PBS) twice. In the next procedure, MTT (Sigma, Germany) solution were instilled into each well and incubated for 3 h in the same condition. Then, 150 µL of dimethyl sulfoxide (DMSO) (Sigma, Germany) was added to each well after the complete removal of the supernatants. The absorbance microplate reader (Epoch, USA) was applied for determining the optical density (OD) of the solution at 570 nm with the wavelength of 630 nm serving as a reference when formazan crystals were completely dissolved. Each procedure was conducted three times. The subsequent equation was used to determine the percentage of viable cells.

$(OD_{test}/OD_{control}) \times 100$

2.3. Evaluating adMSC-secretome effect on JAk2, STAT3, PI3k, and mTOR gene expressions in cancerous cells

For studying the adMSC-secretome effect on expression rates of JAk2, STAT3, PI3k, and mTOR genes in A431 and AGS cell lines, each cancerous cell line was co-cultured with ad-MSCs for 96 h, followed by conducting real-time PCR. Briefly, all of the inserts were removed and cancerous cells were washed twice with PBS after co-culturing of cancerous cell lines with adMSCs for 96 h (as indicated). Then, total RNA from each cancerous cell line was extracted by use of a Total RNA Extraction kit (Parstous, Iran). The quality of extracted RNA (its concentration and purification) was determined by Take3™ spectrophotometer (BioTek, USA) at A260/A280 ratio. Easy cDNA Synthesis kit (Parstous, Iran) was utilized for cDNA synthesis based on manufacturing instructions. In the next step, the expression rates of genes (JAK2, STAT3, PI3k, and mTOR) in cancerous cell lines were determined by quantitative real-time PCR applying RealQ Plus 2x Master Mix Green High ROX™ (Ampliqon, Denmark) based on manufacturing instructions. Table 1 indicates the list of primers. GAPDH

was considered as the reference gene here. The experiment was conducted in triplicate for each sample. The Livak ($2^{-\Delta\Delta C_T}$) method was employed to determine the gene expression levels (Livak and Schmittgen, 2001).

Table 1. Primer set sequences for quantitative real-time PCR

Gene name	NCBI reference sequence	Primer sequence ('5' - '3')	Product size (bp)	Annealing temperature (°C)
JAK2	NM_004972.4	Forward ATCTGGGGAGTATGTTGCAGAA	124	60
		Reverse GTTGGGTGGATACCAGATCCTTT		
STAT3	NM_139276.3	Forward GAATCACGCCTTCTACAGACT	125	60
		Reverse TTCCGGACATCCTGAAGGT		
PI3k	NM_006218.4	Forward CATGGAGGAGAACCCTTATGTGA	114	60
		Reverse AGCACGAGGAAGATCAGGAATG		
mTOR	NM_004958.4	Forward CAACAAGCGATCCCGAACGA	78	60
		Reverse CCAAGTTCCACACCGTCCAA		
GAPDH	NM_002046.7	Forward TGAAGGTCGGAGTCAACGG	148	60
		Reverse TGGGTGGAATCATATTGGAACA		

2.4. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analyses were conducted by use of GraphPad Prism Version 9 software (GraphPad Software, USA). The difference between test and control groups in each experiment was calculated utilizing an independent Student's t-test and one-way ANOVA (tukey post-test). A *p*-value less than 0.05 was considered to be a statistically significant difference.

3. Results

3.1. adMSC-secretome showed cytotoxic effect against AGS cell line with no cytotoxicity against A431 cell line

For examining the cytotoxicity of adMSC-secretome on cancerous cell lines, an MTT assay was performed after a 96-h co-culturing of each cell line with adMSCs. As shown in Fig. 1a, the percentage of viable cells in the test group of AGS cell line (76.71 ± 3.97%) is significantly reduced as compared to its control group (*p*-value < 0.0001), while cell viability increases in test group of A431 cell line by 30.12 ± 5.97% (in comparison with control, *p*-value < 0.001) (Fig. 1b), indicating cytotoxicity of adMSC-secretome against AGS cell line.

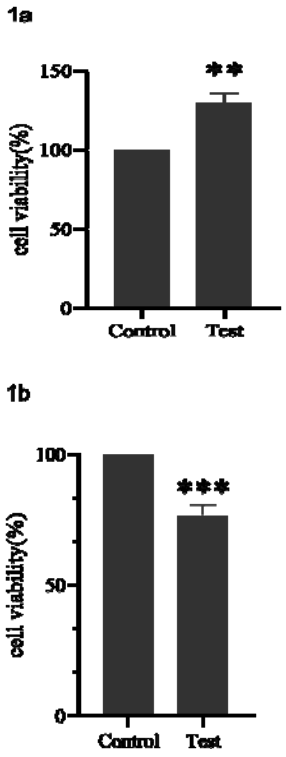

Figure 1. The percentages of viable cells in A431 (1a) and AGS (1b) cell lines after co-culturing with adMSCs; Values are presented as Mean ± SD; ** Significantly different compared to the control group (*p*-value < 0.001); *** Significantly different compared to the control group (*p*-value < 0.0001).

3.2. adMSC-secretome downregulated expression rates of JAk2, STAT3, PI3k, and mTOR genes in both cancerous cell lines

In order to analyze the changes in expression rates of genes related to JAK2/STAT3 and PI3k/AKT/mTOR signaling pathways in each cancerous cell line, real-time PCR was conducted following co-culturing of either A431 or AGS cell lines with adMSCs. As illustrated in Fig. 2, the expression rates of JAk2, STAT3, PI3k, and mTOR genes in both co-cultured cancerous cell lines with adMSCs are significantly reduced compared to control groups (p-value < 0.05 for all of the genes in both cell lines in comparison with control).

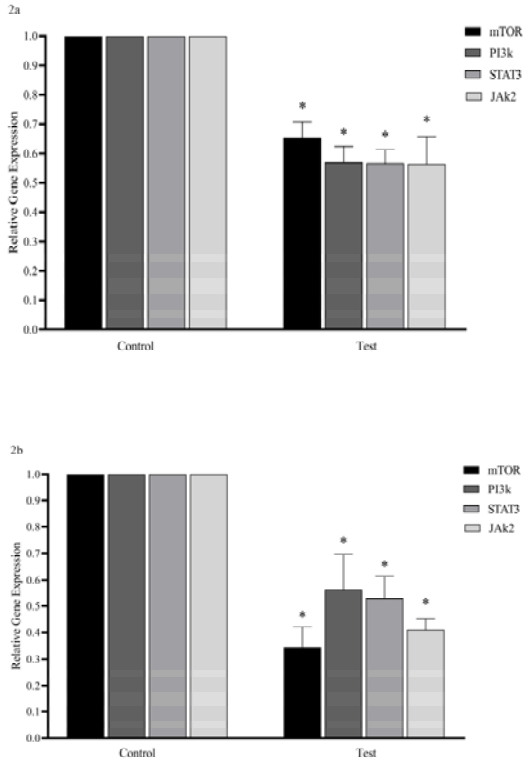

Figure 2. Relative gene expression of mTOR, PI3k, STAT3, JAk2 of in A431 (2a) and AGS (2b) cell lines after co-culturing with adMSCs; Values are presented as Mean ± SD; * Significantly different compared to the control group (p-value < 0.05).

4. Discussion

Stem cells and their secretomes could be regarded as effective cancer therapeutic agents with minimum side effects. Based on the evidence, the MSC and its secretome comprising a diverse range of cytokines and other bioactive molecules can impede the growth of numerous cancer cell types such as cholangiocarcinoma (Jantalika *et al.*, 2022), hepatocellular carcinoma (Hou *et al.*, 2014; Opo *et al.*, 2023; Tang *et al.*, 2016), prostate cancer (Sousa *et al.*, 2023; Takahara *et al.*, 2014), ovarian (Kalamegam *et al.*, 2019), leukemia (Zhu *et al.*, 2009), breast cancer (Pakravan et al,. 2017), and melanoma (Ahn *et al.*, 2015). In addition, some studies revealed that the cancerous behavior in tumor cells is promoted by MSCs (Chen *et al.*, 2019; Halpern *et al.*, 2011; Spaeth *et al.*, 2009; Suzuki *et al.*, 2011; Xu *et al.*, 2009; Yan *et al.*, 2012; Zhu *et al.*, 2006). Additional investigations are required to thoroughly define the safety and effectiveness of MSC/its secretome and determine its mechanisms of action, considering the opposite effects of MSC or its secretome against cancerous cells. This study aims to evaluate the cytotoxicity of adMSC secretome against gastric adenocarcinoma (AGS) and epidermoid carcinoma (A431) cell lines and determine its inhibitory effect on JAK/STAT and PI3k/AKT/mTOR, as two main cancer signaling pathways.

To this aim, each cancerous cell lines (either AGS or A431) were co-cultured with adMSCs for 96 h, followed by an MTT assay. The results represented that MSC secretome affected AGS and A431cell lines differently, while MSC secretome reduced viable cells in AGS cell line with no cytotoxic effect against A431 cell line. These results are in line with those reported before and confirm the opposite behavior of MSCs towards different cancerous cells. Some researchers (e.g., Goldstein *et al.*, 2010; Sousa *et al.*, 2023) revealed that the proliferative effect of MSCs varied depending on the type of tumor, indicating their different responsiveness toward external stimuli. Besides the type of tumor or cancerous cell line, some other factors may explain such discrepancies in MSC behavior, source of MSCs, co-culturing method, treatment duration, and concertation of MSC conditioned media. The dose- and time-dependent effects of MSCs were shown in different studies (Jantalika *et al.*, 2022; Opo *et al.*, 2023; Sousa *et al.*, 2023).

In addition, this study seeks to determine the effect of adMSC secretome on the expression rates of genes such as JAk2, STAT3, PI3k, and mTOR related to JAK2/STAT3 and PI3k/AKT/mTOR signaling pathways by conducting real-time PCR. Based on the literature review, any alterations in the mRNA level of a gene could be related to the same change in its protein level (Creighton *et al.*, 2010; Deng *et al.*, 2015; Fu *et al.*, 2021; Riquelme *et al.*, 2016). The results represented that adMSC secretome significantly downregulated the expression rates in all of the indicated genes in both cell lines, resulting in inhibiting JAK2/STAT3 and PI3k/AKT/mTOR signaling pathways. Jantalika *et al.* argued that cholangiocarcinoma cell lines underwent apoptosis due to the suppression of JAK2/STAT3 signaling pathways by human chorion-derived mesenchymal stem cells (Jantalika *et al.*, 2022). The findings of this study are consistent with those reported by Sousa *et al.* (2023), indicating that umbilical cord-MSC secretome decreased PI3K/AKT activation in prostate cancer cell lines.

Based on some studies, excessive activation of JAK/STAT and PI3k/AKT/mTOR signaling pathways leads to proliferation, metastasis, and survival in different types of tumor cells (Fattahi *et al.*, 2020; Mengie Ayele *et al.*, 2022; Rah *et al.*, 2022; Tewari *et al.*, 2022). Thus, it is expected that the inhibition of the above-mentioned signaling pathways reduces the cancerous cell viability. The gene downregulation in A431 cell line increased cell viability although downregulation in JAk2, STAT3, PI3k, and mTOR genes might be responsible for the cytotoxic effect of adMSC secretome against AGS cell line. Therefore, it is hypothesized that JAK/STAT and PI3k/AKT/mTOR are not considered as the main proliferative signaling pathways in epidermoid adenocarcinoma and cannot be regarded as candidate

therapeutic targets, while they may be proper targets for therapeutic agents in gastric carcinoma. The aforementioned claim means that activation of some other proliferative signaling pathways in A431 cell line might compensate for the inhibitory effect of MSCs on JAK2/STAT3 and PI3k/AKT/mTOR, leading to increased cell viability in A431 cell line. In addition, Fadhal (2023) reported that the functions of signaling proteins may differ based on the type of cancerous cell.

It is worth to highlight that the simultaneous inhibitory effect of adMSC secretome on both signaling pathways is significant. Further, Yeh *et al.* (2013) asserted that the single inhibitory effect of MSCs on PI3k/AKT/mTOR pathway led to manifestation of metastatic and aggressive tendencies in tumor cells. However, its dual inhibitory effects on both JAK2/STAT5 and PI3k/AKT/mTOR signaling pathways play main role in reducing cancer cell number, growth of tumor and metastasis, as well as increasing survival *in vivo*. Finally, Janku *et al.* (2014) found that combinatorial targeting of two signaling pathways led to an enhanced therapeutic response.

5. Conclusion

This study reported the dual inhibitory effect of adMSC secretome on JAK2/STAT3 and PI3k/AKT/mTOR signaling pathways in epidermoid carcinoma and gastric adenocarcinoma cell lines, confirming the cell line-dependent manner of MSCs with no cytotoxicity against A431 cell line, considering the cytotoxicity of MSCs against such cancerous cell lines. adMSC secretome showed cytotoxic effect against AGS cell line. Furthermore, the downregulation of all of the studied genes related to JAK2/STAT3 and PI3k/AKT/mTOR signaling pathways in both cell lines revealed that the role and significance of each signaling pathway may differ based on tumor type. The aforementioned data confirm the selective cytotoxicity of MSCs against different cancerous cell lines, indicating that determining the precise effects of MSCs on signaling pathways in each type of cancer is needed and can be considered as a guide for utilizing MSC and its secretome in further *in vivo* studies and clinical trials.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Author contribution

SD performed the study conception and design. Material preparation, experiments, and data collection were performed by GM, AB, SN, MA, FK, and MT. The data were analyzed by MT, EM, and SD. The first draft of the manuscript was written by GM, EM, and SD. All of the authors reviewed the manuscript.

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