Summary

The effect of extracellular vesicles derived from mesenchymal stem cells on the suppression of biological activity of ovarian cancer stem cells

Ovarian cancer is one of the seven most common cancers in women in the world. Due to the lack of symptoms in the initial stages of the disease, patients are diagnosed in an advanced stage, usually with metastases to other organs. Metastasis of ovarian cancer are commonly associated with ascitic fluid accumulation in the peritoneal cavity. The choice of therapeutic method depends on several factors, including the histological type of cancer, its advancement, and the patient's clinical condition. A commonly used method is chemotherapy based on cytostatic drugs (platinum or its derivatives) followed by surgical removal of the tumor. Therapy failures are related to the presence of a rare population of cancer stem cells (CSCs) in the tumor microenvironment, which are resistant to commonly used chemotherapy drugs and radiotherapy. Therefore, there is an urgent need to develop cellular models enabling research on the biology of CSCs and the identification of biological or pharmacological factors that can inhibit the biological activity of CSCs.

Mesenchymal stem cells (MSCs) represent a heterogeneous population of multipotent cells that reside in most human tissues and are responsible for maintaining organ homeostasis. Research on the biology of MSCs documents that they may become promising tools in clinical applications in tissue regeneration processes due to their high proliferative potential, anti-inflammatory and immunomodulatory properties. Moreover, MSCs also release various bioactive molecules in the form of soluble factors, exosomes and microvesicles (MVs), which act in the tissue microenvironment as mediators of cell-cell communication, exerting paracrine effects. Numerous studies on cancer have shown conflicting results related to the anti-tumor or pro-tumorigenic activity of MSCs and their derivatives (exosomes and microvesicles).

This doctoral dissertation presents research on the anticancer activity of MVs, derived from immortalized MSCs obtained from adipose tissue (HATMSC2-MVs), in ovarian cancer cells.

The objectives of the dissertation:

1) Characterization of MVs derived from immortalized adipose tissue mesenchymal stem cells (HATMSC2-MVs); 2) Assessment of the impact of HATMSC2-MVs on the biological activity of ES-2 and OAW-42 ovarian cancer cells; 3) Characterization of primary ovarian cancer cells derived from postoperative ovarian cancer tissue and ascitic fluid; 4) Assessment of the impact of HATMSC2-MVs on the biological activity of primary ovarian cancer cells using a 2D and

3D model; 5) Analysis of pro-apoptotic and anti-apoptotic proteins in HATMSC2 cells and HATMSC2-MVs; 6) Assessment of the phenotype and biological properties of primary cells from ascites fluid (OvCa3 A and OvCa7 A) and the immortalized cell lines OvCa3 A hTERT and OvCA7 A hTERT derived from primary cells.

The doctoral dissertation consists of a series of three thematically related works, published in a peer-reviewed journal from the JCR list.

The first publication (*IJMS 2020, 1;24(21):15862*) characterized microvesicles derived from immortalized MSCs obtained from adipose tissue (HATMSC2-MVs). A homogeneous population of HATMSC2-MVs with an average size of approximately 450 nm was obtained, which was confirmed by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM). HATMSC2-MVs expressed CD73, CD90, CD105, HLA-ABC markers and lack of expression of CD45 and HLA-DR markers, comparable to the phenotype of parental HATMSC2 cells.

Further in the study, the influence of HATMSC2-MVs on the biological activity of ovarian cancer cells representing: clear cell carcinoma (ES-2) and cystadenocarcinoma (OAW-42) was assessed. The effect of HATMSC2-MVs on the proliferative activity, cell cycle, cell survival, phenotype and secretory profile of cancer cells was investigated. Before biological activity tests, the internalization of HATMSC2-MVs into cells of the ES-2 and OAW-42 ovarian cancer lines was confirmed using flow cytometry and fluorescence microscopy methods. In functional tests, HATMSC2-MVs inhibited cell proliferation (MTT test) and induced cancer cell death by apoptosis and/or necrosis, as demonstrated by flow cytometry. HATMSC2-MVs did not change the phenotype of ES-2 and OAW-42 cells. Internalization of HATMSC2-MVs into ES-2 and OAW-42 cells led to an increase in the secretion of antitumor factors (e.g., IL-2, IL-15, IFN-γ) by tumor cells and a decrease in the secretion of tumor growth-promoting factors (e.g., VEGF, IL -8, GRO-alpha) as demonstrated by analysis of protein membranes.

The second publication (*IJMS 2023, 30;21(23):9143*) presents the results of the characterization of primary ovarian cancer cells derived from postoperative tissue and ascitic fluid and the assessment of the impact of HATMSC2-MVs on the biological activity of primary ovarian cancer cells in a 2D and 3D model. The presence of bioactive factors regulating apoptosis in HATMSC2 and HATMSC2-MVs cells was also examined using protein membranes. Cell phenotype analysis was performed using flow cytometry, microscopic imaging and real-time PCR. It has been shown that primary ovarian cancer cells from postoperative tissue and ascitic fluid are a heterogeneous population expressing MSCs markers (CD73, CD90, CD105) and CSCs markers (CD24, CD44, CD133, ALDH1, c-kit) at various levels. Moreover, cancer cells

expressed markers for Cancer-Associated Fibroblasts (CAFs) (PDGFRa, FAP), markers of Epithelial to Mesenchymal Transition (EMT) (Snail, vimentin) and markers responsible for maintaining pluripotency (Oct4, Sox2, Nanog) at protein level. Moreover, primary ovarian cancer cells expressed transcripts responsible for pluripotency (Oct4,Sox2) and proto-oncogenic transcripts p53, p21, c-myc at various levels of expression. In functional tests, HATMSC2-MVs influenced the decrease in metabolic activity and had no effect on the migratory activity of primary cells. As in the case of ES-2 and OAW-42 cell lines, treatment of primary tumor cells with HATMSC2-MVs induced apoptosis and/or necrosis. In the last stage of the work, spheroids derived from primary ovarian cancer cells were created and characterized for the presence of CSCs markers (CD133, CD44, CD24). Differences in the expression of the tested CSC markers were observed between spheroids from postoperative tissue and ascitic fluid. In spheroids from postoperative tissue, the expression of the CD133 marker was lower compared to spheroids from ascitic fluid. On the other hand, CD24 levels were higher in spheroids from postoperative tissue compared to spheroids from ascites. However, the CD44 marker was present at similar levels for both types of spheroids. The influence of HATMSC2-MVs on the decrease in the survival of cells forming spheroids was demonstrated. The study also shows differences in the expression of the analyzed factors regulating apoptosis between HATMSC2-MVs microvesicles and HATMSC2 parental cells. Out of the 43 bioactive factors tested, 15 pro-apoptotic factors were present at higher levels in HATMSC2-MVs (e.g. bad, BID, BIM, caspase 3, cytochrome c, TRAIL-R1, and TRAIL-R2) than in parental HATMSC2 cells.

In the third paper (*IJMS 2024, 25(10):5384*), the phenotype and biological properties of primary cells from ascites fluid (OvCa3 A and OvCa7 A) and the immortalized cell lines OvCa3 A hTERT and OvCa7 A hTERT derived from them were compared. Phenotype analysis using flow cytometry and microscopic imaging showed differences between primary cells and the immortalized lines obtained from them, in the expression of markers of ovarian tumor cells (Pax8, p53), of epithelial origin (CA-125, cytokeratin 8), MSCs phenotype (CD73, CD90, CD105), CSCs (CD133, CD24, c-kit), CAFs (PDGFRa, FAP), EMT (Snail, vimentin) and markers of hematopoietic cells (CD34, CD45) and those responsible for maintaining pluripotency (Oct4, Sox2, Nanog). An important observation is that the majority of OvCa7 A hTERT cells have the characteristics of CSCs expressing CD133 + and were positive in terms of the presence of the ovarian cancer markers Pax8, p53, CA-125, and cytokeratin 8. In turn, the OvCa3 A hTERT cell line does not express CD133 and is distinguished by the presence of

the marker CD73, higher CD105 expression and lower c-kit expression compared to OvCa7 A hTERT)

The obtained cell lines expressed transcripts responsible for maintaining pluripotency (Oct4, Sox2) and proto-oncogenic ones (p53, p21 and c-myc) at various expression levels, which were analyzed using the real-time RT-PCR method. Functional tests showed that metabolic activity (MTT test) and migratory activity (crack closure test) were higher in both immortalized cell lines, OvCa3 A hTERT and OvCa7 A hTERT, compared to primary cells. Aldehyde dehydrogenase 1 (ALDH1) activity was lower in primary cells compared to the corresponding immortalized lines. Moreover, ALDH1 activity in OvCa7 A hTERT cells was more than 3-fold higher compared to OvCa3 A hTERT cells. To assess the presence of CSCs in the spheroids, the expression of CD133 and CD44 markers was analyzed using flow cytometry. No CD133 cells were detected in spheroids from OvCa3 A and OvCa3 A hTERT cells. For spheroids created from OvCa7 A hTERT cells, the CD133-positive cell population was higher compared to primary OvCa7 A cells (12% vs. 2%, respectively). There were no differences in the expression of the CD44 marker for spheroids created from primary and immortalized cells..

Research carried out as part of the doctoral dissertation showed that the treatment of ES-2 and OAW-42 ovarian cancer cells with HATMSC2-MVs leads to decrease in proliferative activity, induces cell death by apoptosis and/or necrosis, and increases the secretion of anticancer factors and reduces the secretion of factors promoting cancer growth. The results obtained on cell lines were confirmed in studies on primary ovarian cancer cells obtained from postoperative tissue and ascites fluid, where a decrease in metabolic activity, decreased cell survival, induction of apoptosis and/or necrosis in the 2D model and a decrease in cell survival in the 3D model after HATMSC2-MVs treatment were observed. The presence of the population of cells expressing CSCs markers (CD133, CD24, CD44, c-kit (CD117) and ALDH1), and markers responsible for maintaining pluripotency (Oct4, Sox2, Nanog) was also showed in primary ovarian cancer cells isolated from postoperative tissue and ascites fluid. The last stage of research showed that a plasmid carrying the hTERT gene can be used to immortalize primary ovarian cancer cells. The immortalized OvCa7 A hTERT CD133-positive cell line, expressing Pax8 and p53 markers and high ALDH1 activity, may be a useful tool in research on the biology of CSCs and the development of new therapeutic strategies targeting CSCs.