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Effect of the Secretome of Mesenchymal Placenta Stem Cells on the Functional Properties of Lewis Lung Carcinoma Cells In Vitro

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Abstract—This paper concerns the effect produced by the components of a conditioned medium (K-medium), in which mesenchymal human placenta cells (hP-MSC) are cultivated, on the characteristics of Lewis lung carcinoma (LLC) cells in the culture. It is shown for the first time that the K-medium (secretome) components have a prooncogenic effect on LLC cells as evidence by an increase in cell survival rates, LLC cell proliferation stimulation, and a decrease in the level of apoptotic cells. The effect of the K-medium on the adhesion characteristics of LLC cells in the process of their monolayer growth and migration from 3D-cultures is also demonstrated. When the hP-MSC secretome interacts with the cultured LLC cells, the production of proinflammatory cytokines TGF β and Il-6 is observed to grow. At the same time, the proangiogenic factor VEGF remains almost at the same level. Similar changes in the microenvironment during the interaction of mesenchymal and tumor cells may underlie various prooncogenic effects observed in our previous studies with different MSC inoculation methods during the development and metastasis of Lewis lung carcinoma in vivo.

Keywords: Lewis lung carcinoma (LLC) cells, mesenchymal stem cells, cryopreserved human placenta, conditioned medium, TGF β, Il-6, prooncogenic effects

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INTRODUCTION

Mesenchymal stem cells (MSCs) represent a subpopulation of pluripotent stem cells with the capability for self-repair and multipotent differentiation (Shi, 2017). The role of mesenchymal stem cells in enhancing such tumor-associated functions as inflammation, immunosuppression, tumor growth, angiogenesis, and metastasis has been proven (Cuiffo and Karnoub, 2012; Li, 2019; Zhang, 2021). The mutual effect of mesenchymal stem and tumor cells is mediated by different humoral factors secreted into the microenvironment, including a number of pro- and anti-inflammatory cytokines and growth factors (Goel and Mercurio, 2013; Tanaka, 2014; Liu, 2018; Patel, 2023; Schaperand Rose-John, 2015). MSCs exhibit affinity to tumor cells; enhance proliferation, motility, invasion, and metastasis; stimulate angiogenesis; promote tumor desmoplasia; and suppress antitumor immune responses. Invasion and metastasis are the main characteristics and important markers of malignant tumors. The first stage of these processes is the epithelial-mesenchymal transition (EMT) when differentiated epithelial cells acquire mesenchymal properties (Romano, 2020). The results of many studies show that MSC participate in the control over tumor growth and metastasis in different ways, in particular, in the contact interaction of cells to accelerate the progress of tumors. However, other data show that oncogenic signs are decreased under the influence of mesenchymal stem cells (Suzuki, 2011). For this reason, a promising target of antitumor therapy may be MSC secre-

tome components (proangiogenic, anti-inflammatory humoral factors, etc.).

The multidirectional effects of human placenta MSCs (hP-MSC) on the development and metastasis of human lung carcinoma transplanted to Black C57 mice depending on the method of MSC inoculation into the organism of animals were revealed by us earlier (Stepanov et al., 2024). In particular, local (immediately into a tumor) and systemic intravenous hP-MSC administration when the growth, metastasis, and angiogenesis of tumors were different in both quantitative and qualitative characteristics were used (Stepanov et al., 2024; Ostrovska et al., 2024). The objective of the submitted study was to analyze the possible mechanisms of such ambiguous hP-MSC effects on a tumor by investigating the effect of the K-medium of these cells on the specific features of the adhesion, proliferation, and migration of LLC cells in a culture.

MATERIALS AND METHODS

LLC Cells

The LLC strain was kindly provided by the National Bank of Cell Lines and Tumor Strains of the Kavetsky Institute of Experimental Pathology, Oncology, and Radiology of the National Academy of Sciences of Ukraine. LLC cells were cultivated in RPMI-1640 medium (Sigma, United States) with addition of FBS (10%, Sigma, United States), glutamine (1%), and an antibiotic antimicotic (1%, Thermo Fisher Scientific, United States) at 37°C in a 100% humidity atmosphere with 5% CO_2.

Preparation of hP-MSC

hP-MSC were derived from a full-term placenta (physiological delivery after clinically normal pregnancy), which was treated by the original selection, transportation, and cryofreezing method as described earlier (Navakauskienė et al., 2023). To obtain hP-MSC, defrosting was carried out on a water bath at +38 to +40°C until a liquid phase appeared (0°C) and, further, DMSO was gradually removed by slowly adding a Hanks solution to the tissue. The defrosted tissue was cut into small pieces and, to obtain the growth of cells from explants, the fragments were transferred into cultural flasks with a cultural medium based on α-MEM (Sigma, United States) with addition of 15% bovine fetal serum (Sigma, United States). hP-MSC was cultivated at 37°C and 5% CO_2 for four passages, and the medium was replaced every 3 days. The cells were characterized by the capability for differentiation in osteogenic, adipogenic, and chondrogenic directions and the expression of surface markers by the minimal ISCT criteria (Dominici, 2006; Navakauskienė et al., 2023).

Derivation of the K-Medium

The K-medium was derived for hP-MSC at the fourth cell cultivation passage. To accomplish this, the MEM medium (Sigma, United States) free from embryonal serum was added to the cells, which formed almost 80% of monolayers. The resulting medium, in which hP-MSC were grown, was sampled in the amount of 50 mL to be centrifuged at 3000 *g* (for removal of cellular debris) and stored at +4°C.

Determining the Level of Secretion for Proangiogenic Factors

In the medium of LLC cells after 2-day cultivation with the K-medium of hP-MSC and the control sample (MEM), the level of production for the endothelial cell growth factor (VEGF), transforming growth factor (TGFβ-1), and interleukin (IL-6) was determined by using enzyme immunoassay kits according to the recommendation of the manufacturer (OmnimAbs, United States).

Studying the Effect of the K-Medium of hP-MSC on the Survival Rate, Adhesion, and Migration of LLC Cells In Vitro

Studies were carried out on LLC cells after they were planted into 96- and 12-well plates (Costar, United States) and 70–80% of monolayer were attained and during spheroid growth induced with 0.2% carboxymethyl cellulose (Sigma, United States). The K-medium was titrated from hP-MSC with respect to LCC in vitro at dilution ratios from 1 : 1 to 1 : 1024. LLC cells were cultivated in RPMI-1640 medium (Sigma, United States) with addition of 10% embryonal bovine serum. After a nearly full monolayer was attained, the hP-MSC K-medium was added, and serum-free MEM (Sigma, United States) was poured to the control sample to cultivate for 2 days, after which the survival rate and adhesion and migration potential of cells were determined.

Determining the Survival Rate of LLC Cells In Vitro

The quantitative analysis of the ratio between living and dead cells was carried out after their staining with a 0.4% Trypan Blue solution (Sigma, United States) prepared from 0.1M PBS with pH 7.2. To the suspension of cells, an equal volume of 0.4% Trypan Blue solution was added and, after 5 min, the cells within five large squares of the Goryaev chamber were counted. The average quantity of cells and the quantity of cells in 1 mL were determined taking into account the selection of cells and the volume of incubation. The cells were counted after cultivation of LCC cells with the complete replacement of the K-medium of hP-MSC and at dilution ratios of 1 : 1, 1 : 4, 1 : 16, 1 : 64, and 1 : 256. The refence sample was LLC cells cultivated in MEM.

Determining the Cell Cycle and Apoptosis of LLC

The cell cycle distribution and the level of apoptosis were determined by flow cytometry (Nicoletti, 1991; Garmanchuk et al., 2019). LLC cells cultivated for 2 days in MEM (control sample) and the K-medium of hP-MSC were analyzed. The suspension of cells (5×10^5) was settled by centrifugation at 400*g* for 5 min and washed twice with a phosphate salt buffer (PBS, pH 7.2). The cells were resuspended in PBS (200 μL) with further dilution with 0.1% Tritone (300 μL) in a citrate buffer (pH 6.8). In 1 min, ribonuclease (10 μL, Sigma, United States) and iodide propidium (10 μL, Sigma, United States) were added to the samples. The samples were incubated for 10 min at 37°C and 30 min at room temperature in darkness, centrifuged at 400*g* for 10 min, and the supernatant was further removed. The precipitate was fixed by adding a 0.4% formalin solution (400 μL) in PBS. Measurements were carried out on a FACS Calibr flow cytometer (Becton-Dickinson, United States) with an argon laser at 488 nm and 582/42 nm. Flow cytometry data were analyzed by using the Mod Fit LT 3.0 software (BDIS, United States). The level of apoptosis based on the loss of DNA due to its internucleosome fragmentation was determined in the hypodiploid zone.

Estimating the Adhesive Characteristics of Cultivated Cells

The adhesivity of cells was estimated under the conditions of incubation with the K-medium of hP-MSC and in the control sample and checked for substrate dependence by the quantity of cells that adhered to the surface of a 24- or 96-well plate in 2 days after incubation. The adhesivity of LLC cells was estimated after their staining with crystalline violet with further dissolution in DMSO from the optical absorption ($\lambda =$ 570 nm) measured on a Labsystems Multiskan MS spectrophotometer (Synergie Biotec, United States) as described in Golovynska (2021).

Migration Test

Migration test under the influence of the K-medium of hP-MSC was carried out by the ability of LLC cells to adhere and spread on a 3D-culture substrate. For this purpose, 3D-spheroids were induced on a lowly adhesive substrate with addition of 0.2% carboxymethyl cellulose (Sigma, United States). To the LLC cells in 3D-growth, the K-medium of hP-MSC was added, and MEM was poured into the control sample. After 2 days of cultivation, the surface area of spheroids and the surface area of cells spread on the substrate were determined by using the ImageJ 1.45 software (NIH, United States) from the digital microphotos taken with a Canon camera and a Carl Zeiss Axiovert 40 inverted optical microscope (Germany).

Statistical Analysis

The statistical analysis of obtained study results was carried out by using the Statistica 6.0 and Microsoft Excel software. The normality of the distribution of quantitative experimental data was estimated, and the reliability of revealed changes was assessed by the Student's *t*-test, whose reliability of values was accepted at $p < 0.05$. Morphometric characteristics were calculated by the AxioVision and ImageJ applications. The obtained results were presented in the form $M \pm m$ (mean value ± standard error of mean).

RESULTS AND DISCUSSION

Previously, the hP-MSCs transcript was analyzed to reveal an increase in the expression of cytokines *IL34*, *IL6*, *CXCL8*, *EDN1*, *IL17D*, *CSF2*, *IL32*, *IL11*, and *IFNA1* and a decrease in the expression of angiogenesis-associated genes *ANGPTL1*, *ANGPTL2*, *PDGFB*, and *PDGFD* for hP-MSC (Navakauskienė et al., 2023). However, the data obtained in the previous studies (Stepanov et al., 2023; Ostrovska et al., 2024) on the occurrence of growth enhancement, metastasis, and angiogenesis after hP-MSC administration, both local immediately into an LLC tumor in the femoral muscle of C57 Black mice and systematic intravenous, indicate that not only the hP-MSC cells but also the humoral factors secreted by these cells may participate in these processes. For this reason, the levels of secretion were checked for TGF β, VEGF, and IL-6, which can stimulate the oncogenic properties of LLC cells.

A slight growth was revealed for cytokines TGF β and IL-6 (Table 1), whereas the VEGF levels were almost identical to the control sample.

Despite the tumor suppressive effects, TGF β is one of the most powerful regulators of interaction between tumor cells and the extracellular matrix, as it modulates such processes as cell invasion, immune regulation, and the microenvironment of tumor cells (Massagué, 2008; Liu, 2018); interleukin-6 (IL-6) is a pleiotropic cytokine participating in the regulation of hematological and immune response (Bielins'ka, 2014). IL-6 is generally secreted by stromal cells, but little is known about its certain role in human MSC homeostasis and the role it can play in immune regulation mediated by it (Dorronsoro, 2020). Our results indicate that the incubation of LLC cells with the K-medium of hP-MSC led to a significant increase in the secretion of TGF β (up to 20%, $p < 0.05$) and IL-6 (24%, $p < 0.05$) in contrast to VEGF, the level of which was the same as for the control sample.

Determining the Survival Rate of LLC Cells In Vitro

We revealed in our study that the K-medium of hP-MSC has a stimulating effect on the proliferative

Table 1. Level of TGF β,VEGF, and IL-6 production by LLC cells under the conditions of their cultivation in the control and enriched K-medium of hP-MSC

Growth factor	Incubation conditions	
	LLC (control)	LLC + K-medium of hP-MSC
TGF β, pg/mL	180 ± 8	216 ± 10*
VEGF, pg/mL	90 ± 6	99 ± 9
IL-6, pg/mL	120 ± 7 (100%)	149 ± 10*

* Significant difference as compared to the control sample ($p < 0.05$).

Table 2. Quantity of viable LLC cells and content of dead LLC cells during cultivation for 2 days with the K-medium of hP-MSC at different dilution ratios

Diluted K-medium of hP-MSC	Undiluted K-medium	1 : 1	1 : 4	1 : 16	1 : 64	1 : 256	Control
Concentration of LLC cells	$12.5 \times 10^3 \pm 0.4 \times 10^3$*	$11.8 \times 10^3 \pm 1.1 \times 10^3$*	$12.9 \times 10^3 \pm 1.7 \times 10^3$*	$13.2 \times 10^3 \pm 2.2 \times 10^3$	$9.3 \times 10^3 \pm 1.7 \times 10^3$	$7.9 \times 10^3 \pm 0.9 \times 10^3$	$8.7 \times 10^3 \pm 0.9 \times 10^3$
Content of dead cells, %	9.5*	8.1*	5.4*	14.4*	15.7	18.9	23

* Significant difference as compared to the control sample ($p < 0.05$).

activity and survival rate of tumor cells as compared to the control sample (Table 2).

When LLC cells were cultivated with the K-medium of hP-MSC, both grew by 1.4–1.5 times ($p < 0.05$), whereas the content of dead cells decreased by 2.2–4 times as compared to the control sample after the full replacement of the K-medium of hP-MSC and at dilution ratios of 1 : 1 and 1 : 4 (Table 2).

The obtained data confirm the results of in vivo experiments on the stimulating effect of hP-MSC on the growth of a primary Lewis lung carcinoma tumor and on their inoculation immediately into a tumor as

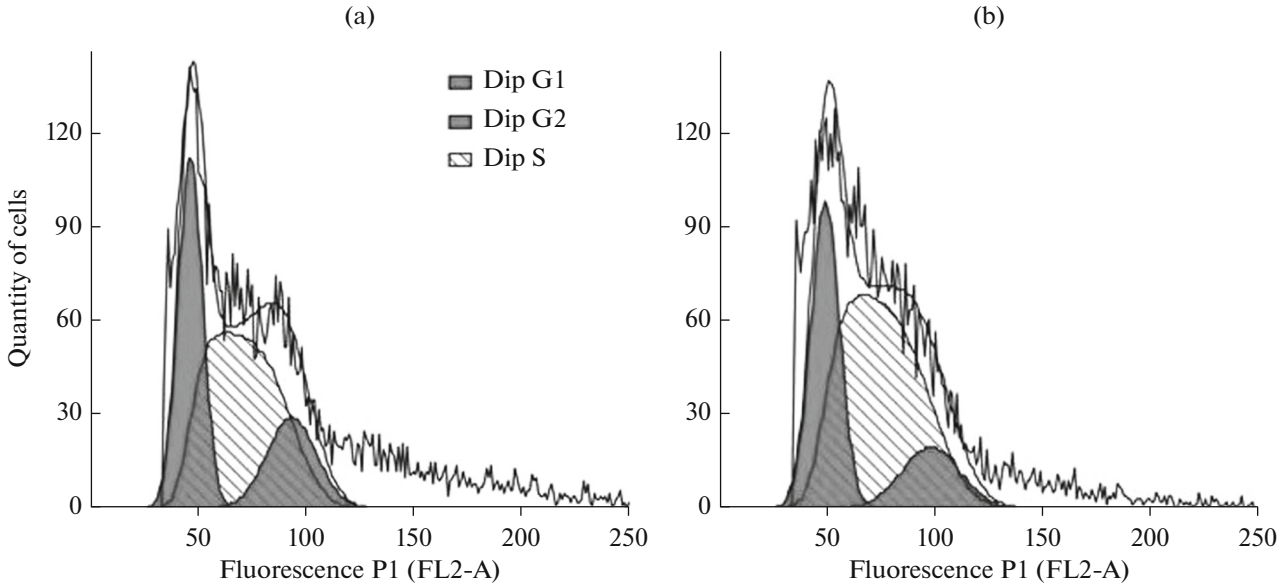

Fig. 1. Cytofluorometric analysis histograms for the LLC cell cycle during monolayer growth under the influence of the K-medium of hP-MSC.

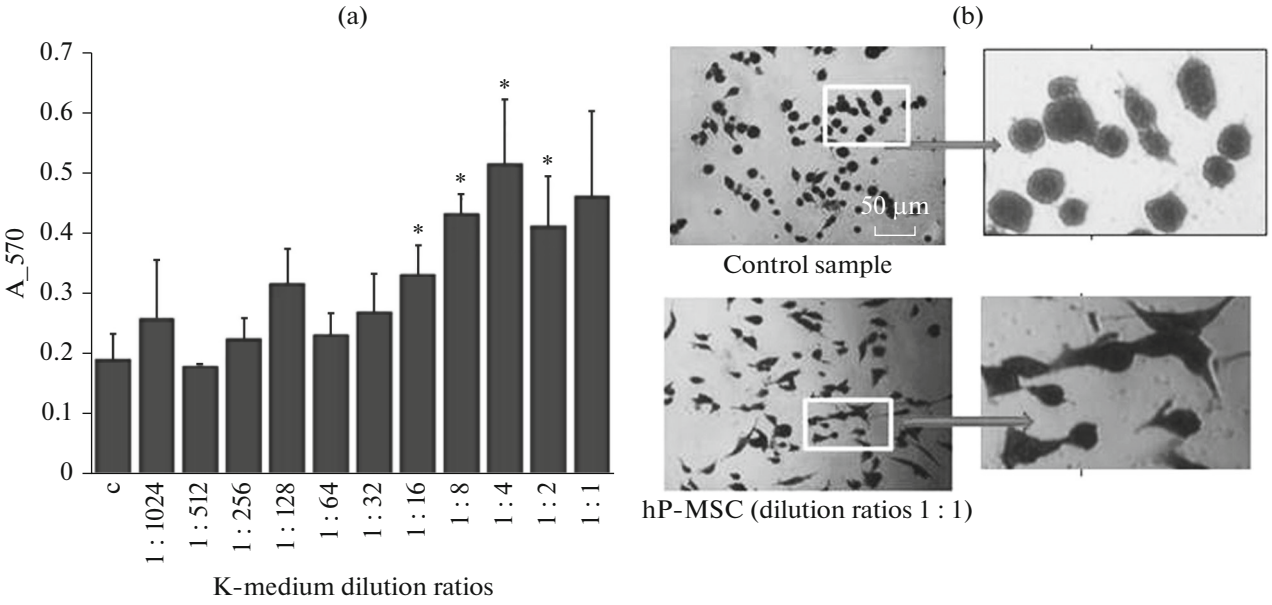

Fig. 2. Adhesion characteristics of LLC cells at different dilution ratios of the K-medium of hP-MSC: (a) colorimetric analysis, (b) morphological analysis (representative photo); * stands for a significant difference as compared to the control sample ($p < 0.05$).

well as on intravenous systemic administration (Stepanov et al., 2024).

Effect of the K-medium of hP-MSC on the Progression of the LLC cell cycle in Monolayer Growth

The stimulating effect on LLC cell proliferation was detected by us in cell cycle analysis. The content of LLC cells in the S-phase of the cell cycle and, correspondingly, their proliferative ability increased under the influence of hP-MSC (Fig. 1, Table 3).

According to the obtained data, the content of cells in the S-phase of the cell cycle grows: thus, under the influence of the K-medium of hP-MSC, the content of cells in the S-phase grows by 17% as compared to the control sample ($p < 0.05$) but decreases by 24% in G0/G1 (Table 3).

The content of cells in the G2/M phase and control sample under the influence of the K-phase of hP-MSC was not essentially different. No pro/antiapoptotic effect of the K-medium of hP-MSC on LLC cells in monolayer growth has been revealed: the level of apoptosis was approximately 15% in both cases. The revealed stimulating effect of the hP-MSC secretome on the proliferative activity of tumor cells in vitro may be promotive for both the growth and survival of a tumor as a whole and the activation of proangiogenic structures formed by them in vivo (Stepanov et al., 2023; Ostrovska et al., 2024).

Estimating the Adhesion Characteristics of Cultivated Cells

When LLC cells were cultivated with the K-medium of hP-MSC, the total adhesive potential was increased.

The cells in the control medium retained a roundish shape with a minimum of appendages, and there no signs of their spread and attachment to the substrate. At the same time, the dilution of the K-medium of hP-MSC at a ratio of 1 : 1–1 : 16 induced an increase in the quantity of cells attached to the substrate (by 1.5 times on average as compared to the control medium (Fig. 2)).

On the one hand, it is considered that an increase in the strength of adhesion corresponds to a decrease

Table 3. Distribution of LLC cells between the cycle phases in the control sample and under the conditions of cultivation with the K-medium of hP-MSC

Cycle phase	G1/G0, %	G2/M, %	S, %
Control LLC	30.39 ± 1.40	17.21 ± 0.61	52.41 ± 0.79
LLC + K-medium of hP-MSC	23.15 ± 4.50*	15.09 ± 2.55	61.76 ± 1.96*

* Significant difference as compared to the control sample ($p < 0.05$).

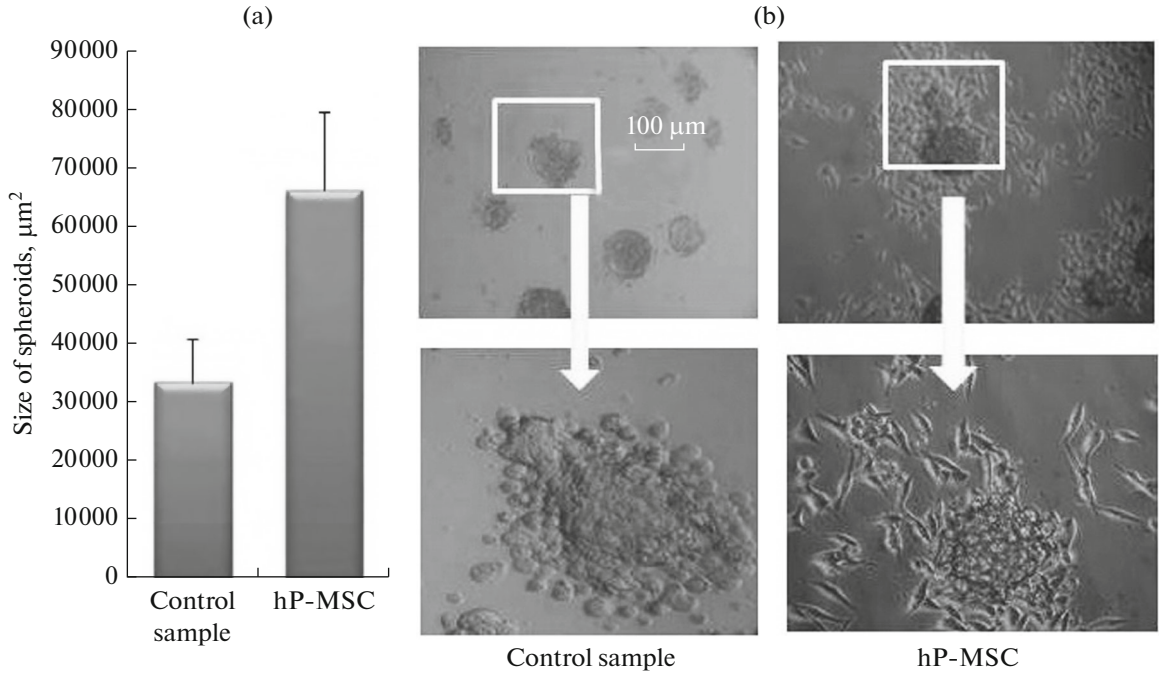

Fig. 3. Sizes of LLC cells spheroids under the influence of the K-medium of hP-MSC: (a) control sample, (b) concentrated K-medium (1 : 1).

in the metastatic potential of tumor cells (Beri et al., 2020). Nevertheless, it can be seen from the microphotos shown in Fig. 2b that the morphological features of LLC cells under the influence of the K-medium of hP-MSC were characterized not only by more pronounced spread and passive attachment but also by active rearrangement in the cytoskeleton and focal contacts as reflected in an increase in the number of appendages and a change in the shape of cells to trapezoids and polygons. Similar rearrangements indicating the growth of focal adhesion processes dynamics make it possible to predict that LLC cells treated with the K-medium of hP-MSC are able to have more pronounced motility and, correspondingly, higher invasion potential, which is one of the manifestations of tumor cells' oncogenicity. Cell adhesion is an important feature of intercellular communication and regulation and has a fundamental significance for the development and maintenance of tissues. Mechanical interaction between a cell and its extracellular matrix may influence and control the behavior and function of a cell (Khalili and Ahmad, 2015). In addition, adhesion is a critical component of malignant transformation, cancer progression, and chemoresistance development. Adhesion molecules allow tumor cells to enter the tissues surrounding a primary tumor and extravasate during metastasis. Moreover, adhesion molecules serve as anchors, which place tumor cells in the immediate proximity to immune and stromal cells (Razi, 2023).

Migration Test

In recent years, tissue-engineered 3D cancer models are widely used in cancer studies. 3D models are more biomimetic in their nature as compared to two-dimensional cell monolayers to reflect the tissue-specific spread of a tumor with attached layers of proliferative migrating cells and hypoxia and necrosis zones (Pape, 2021). Using a 3D-model, it has been shown that the proliferative and migration potential of LLC cells grows under the influence of the K-medium of hP-MSC (Fig. 3).

Thus, it has been shown that the quantity and size of LLC spheroids in the 3D-culture are increased (almost two times), and the cells migrate from spheroids and spread over the substrate to be subjected to proliferation under the influence of the K-medium of hP-MSC, whereas the cells in the control medium hardly migrate from spheroids and do not spread over the substrate.

The analysis of the cell cycle of a 3D LLC culture shows that the content of cells in the S-phase grows by 12% under the influence of the hP-MSC secretome as compared to the control sample (Fig. 4, Table 4).

In addition, for the cells from the control group under 3D conditions, an increase was observed in the apoptotic index (17 vs. 9% under the influence of the K-medium of hP-MSCs) (Fig. 4) and the quantity of dead (24 vs. 5%, respectively).

Hence, on the whole, the components of the K-medium, in which hP-MSC was cultivated, have a

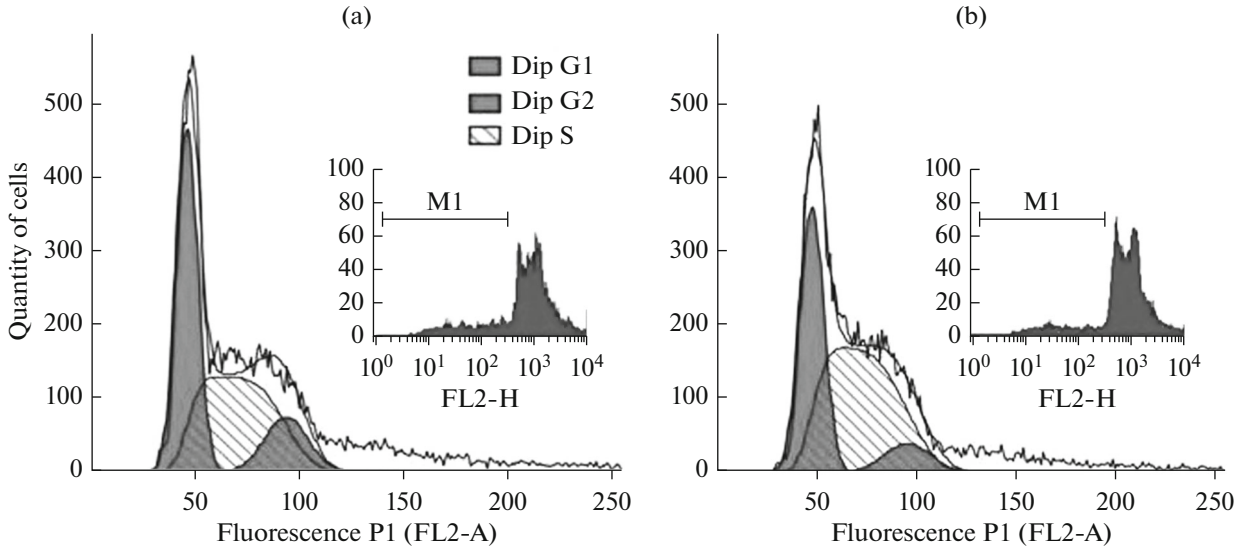

Fig. 4. Cytofluorometric analysis histograms for the cell cycle and the level of apoptosis for LLC cells in 3D cultures under the influence of the K-medium of hP-MSC and in the control sample.

prooncogenic effect on LLC cells in the culture as evidenced by the survival rates of cells: thus, under the influence of the K-medium at dilution ratios of 1 : 1–1 : 16, the percentage of dead cells is decreased by 1.8–2.5 times as compared to the control sample ($p < 0.05$), and the stimulation of proliferation is observed for LLC cells, in particular, the concentration of cells grows by 1.3–1.4 times ($p < 0.05$), and the content of cells in the S-phase of the cell cycle is increased by 1.2 times as compared to the control sample ($p < 0.05$). The modification of the prooncogenic characteristics of LLC cells, such as adhesion to the substrate and migration from 3D-cultures under the influence of the K-medium of was observed. In the secretome of the K-medium of hP-MSC after its addition to the LLC cells, the production of anti-inflammatory cytokines TGF β and Il-6, which play an important part in the modification of a microtumor environment, is observed to grow (Yang, 2010; Alison, 2024). The growth of focal adhesive processes dynamics and migration activity is evidence for the epithelial-mesenchymal transition, which demonstrates the prooncogenic potential of tumor cells (Pawelek, 2008).

The obtained results on the prooncogenic effect from the K-medium of hP-MSC on cultivated LLC cells in vitro confirm the conclusions made in our previous papers about the growth of a tumor and metastatic and angiogenic potentials in LLC carcinoma models in vivo in the presence of hP-MSCs.

According to the literature data (Uder, 2018), different MSC types may exhibit both antioncogenic and prooncogenic properties with respect to tumor cells depending on microenvironment conditions. In our studies, it has been shown for the first time that, under the conditions of the cocultivation of tumor LLC cells with the conditioned human placenta medium MSC, prooncogenic properties are predominant by a number of parameters.

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Table 4. Distribution of LLC cells under 3D growth conditions between the cycle phases and level of apoptotic cells in the control sample and during cultivation in the K-medium of hP-MSC

Cycle phase/apoptosis level	Content of apoptotic cells, %	G1/G0, %	G2/M, %	S, %
Control LLC	17 ± 1.2	42.91 ± 0.72	13.96 ± 0.361	43.13 ± 0.32
LLC+ K-medium of hP-MSC	9 ± 0.71	37.01 ± 1.02*	8.35 ± 061	54.63 ± 1.32*

* Significant difference as compared to the control sample ($p < 0.05$).

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All the studies were conducted in accordance with the principles of biomedical ethics set out in the Helsinki Declaration of 1964 and later amendments to it. The Bioethics Commission of the Institute of Molecular Biology and Genetics of the National Academy of Sciences of Ukraine reviewed the materials of the scientific publication "Effect of the Secretome of Placental Mesenchymal Stem Cells on the Functional Properties of Lewis Lung Carcinoma Cells In Vitro" at Bioethics Commission meeting no. 36 on February 13, 2024. Each participant of the study provided voluntary written informed consent after receiving the explanations of potential risks and benefits as well as the character of the future study.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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