UKRAINIAN JOURNAL OF VETERINARY SCIENCES

2024. Volume 15, No. 2. P. 102-120

Received: 08.02.2024 Revised: 19.04.2024 Accepted: 29.05.2024

DOI: 10.31548/veterinary2.2024.102

UDC 636.09:599.323.41:591.473.3:609.2

Influence of allogeneic mesenchymal stem cells of red bone marrow culture on the development of Lewis lung epidermoid carcinoma *in vivo*

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Suggested Citation:
Kladnytska, L., Tomchuk, V., Velychko, V., Salata, V., & Šengaut, J. (2024). Influence of allogeneic mesenchymal stem cells of red bone marrow culture on the development of Lewis lung epidermoid carcinoma *in vivo*. *Ukrainian Journal of Veterinary Sciences*, 15(2), 102-120. doi: 10.31548/veterinary2.2024.102.

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Abstract. The relevance of this study is conditioned by the widespread use of stem cells in veterinary medicine, a wide range of studies and ambiguous data on the oncoprotective properties of stem cells of different origins. In this regard, the purpose of this study was to investigate the course of the tumour process in Lewis lung carcinoma and the specific features of the effect of allogeneic mesenchymal stem cells of red bone marrow culture on it. The leading approach to investigating this problem was the method of modelling Lewis lung carcinoma in C57BL6 mice and the use of stem cells. The use of allogeneic mesenchymal stem cells from the bone marrow culture of C57BL6 mice with transplanted epidermoid metastatic carcinoma of the Lewis lung contributed to the activation of the tumour process. Under the influence of allogeneic mesenchymal stem cells of red bone marrow culture from Day 14 to Day 24 of the study, the body weight of mice decreased by 7.0-12.1% ($P < 0.05$) compared to the control, the diameter of the primary tumour increased by 1.43-1.51 times ($P < 0.05$), which is conditioned by the activation of primary tumour growth. The number of lymphocytes as producers of vascular growth factor in primary tumour tissue under the influence of allogeneic mesenchymal stem cells of red bone marrow culture significantly increased by 1.47 and 1.52 times on Day 18 of the experiment compared to animals of the control group and placebo ($P < 0.05$), respectively. This promoted angiogenesis in the primary tumour node and metastasis through the circulatory system. After administration allogeneic mesenchymal cells of red bone marrow culture to mice, a larger volume of lung metastases was recorded, which was 41.52 ± 7.9 mm^3 compared to the values in the control and placebo groups, respectively, 17.94 ± 6.59 and 16.43 ± 5.32 mm^3. The morphological picture of the histological sections of the primary tumour of Lewis lung carcinoma confirms all the signs of qualitative and quantitative indicators of its progression. The findings obtained are of both theoretical and practical value for clinical veterinary medicine on the use of allogeneic bone marrow mesenchymal stem cells in tumour processes

Keywords: primary tumour; infiltration; lymphocytes; metastases; vascularisation; C57BL6 mice

Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells with significant potential for differentiation and self-renewal. The therapeutic properties of MSCs are associated with the potential for transdifferentiation, secretion of trophic factors, and immunomodulation. MSCs differentiate into numerous mesenchymal cell lineages, including adipocytes, osteocytes, chondrocytes, pericytes, and fibroblasts. According to H. Yang *et al*. (2022), they are also able to transform into epithelial (endodermal) and neural (ectodermal) lineages, into hepatocytes under certain conditions of in vitro culture, and are involved in maintaining tissue structure and controlling inflammation.

Overall, MSCs have a beneficial effect through immunomodulatory regulation and paracrine mechanisms. Y. Xia *et al*. (2023) report that MSCs have been shown to have a therapeutic effect in many diseases, such as myocardial infarction, liver cirrhosis, limb ischaemia, nervous injury, among others. In general, MSCs maintain tissue integrity as the key regulators of tissue homeostasis. Apart from their normal distribution in individual organs, they accumulate in damaged tissues, promoting tissue regeneration. In response to tissue damage, MSCs are mobilised from their niches by relevant signals and migrate to take part in tissue regeneration and remodelling. Certain cultivation factors and body functioning conditions can increase their intake into the bloodstream and damaged tissues. It is also known that MSCs can be localised together and form the core of blood clots, which leads to circulatory disorders. In addition, L. Frisbie *et al*. (2022) note that MSCs are tumourigenic due to their reproductive properties and the ability to provoke acute or chronic immunogenicity of cells as foreign agents in the body.

The development and progression of a tumour is determined not only by the tumour cells involved, but also by the tumour microenvironment. The cell populations surrounding tumours can interact indirectly or directly with tumour cells and significantly affect their functions and properties. In particular, MSCs play an important role in tumour development, demonstrating various variants of intercellular interaction through cytokines, chemokines, growth factors, which can ultimately trigger the formation of a tumour stem cell pool (TSCP) or the reproduction of new tumour cell populations.

X. Xuan *et al*. (2021) reported that MSCs had multifaceted effects on tumour cells, mainly promoting tumour growth through their role in regulating inflammation and tissue repair. According to G. Zhang *et al*. (2020), mesenchymal stem cells support tumour growth through various mechanisms, including promotion of drug resistance, activation of metastasis, pro-angiogenic effects, enrichment of the cancer stem cell niche, and high immuno-inhibitory properties.

According to G. Guo *et al*. (2021), MSCs also affect the survival and stemness of tumour cells and promote the growth of the tumour vasculature, produce angiogenic factors, and differentiate into pericytes. Mesenchymal stem cells promote tumour cell motility and secrete cytokines (IL-6), chemokines (CXCL1, CXCL2, and CXCL12), and several matrix metalloproteinases that facilitate tumour cell migration. According to C.R. Harrell *et al*. (2021), they have an important immunomodulatory function, which is primarily immunosuppressive. M. Timaner *et al*. (2020) suggest that MSCs can enhance the resistance of tumour cells to anticancer drugs, at least in part, by releasing exosomes containing numerous mediators, including microRNAs, which can alter the properties of tumour cells. Thus, in most studies, MSCs have a pro-tumour effect, but there are studies that have shown that they can also exert an anti-tumour effect by inhibiting Wnt signalling, suppressing angiogenesis, stimulating inflammatory cell infiltration, inducing cell cycle arrest and cause apoptosis. Z. Chen *et al*. (2021) proved that IFN-β is synthesised by adipose tissue-derived MSCs cultured at high cell density, which reduces the growth of breast cancer cells – MCF-7.

Y. Stepanov *et al*. (2021) found that the route of administration of mesenchymal stem cells – intramuscular or intravenous – also has an impact on tumour growth and angiogenesis. Specifically, intramuscular transplantation of placenta-derived stem cells slows down primary tumour growth and angiogenesis compared to the control on Day 15 of the study, while intravenous injection of stem cells stimulates these processes. On Day 23 of the tumour process, no significant changes in tumour growth were observed in animals of both groups, in contrast to angiogenesis, which was slowed or activated, respectively. The number and volume of metastases in both groups of animals was higher than in the control. These findings suggest a bidirectional effect on the development of the tumour process in the early period of development. However, regardless of the route of MSC transplantation, tumour progression, and activation of metastasis are evident.

To obtain enough material for transplantation, scientists have developed methods to activate the proliferation of stem cells during *in vitro* cultivation. Thus, A. Mazurkevych *et al*. (2021) propose to activate proliferation using different concentrations of insulin-like growth factor (IGF-1), growth hormone (rhGH), fibroblast growth factor (FGF-2), and biolaminin 521 LN. The findings obtained upon the use of stem cell growth activators were ambiguous, namely, IGF-1 had a positive effect on proliferative activity, while the effect of rhGH decreased the proliferative index. However, both growth factors did not affect genetic changes in cells.

Thus, considering the numerous and ambiguous results found in the study of the effect of MSCs on the tumour process, the study of carcinogenesis, metastasis, changes in the skeletal muscle of mice with transplanted Lewis lung carcinoma under the influence of allogeneic mesenchymal stem cells of bone marrow culture is a relevant issue.

The aim of the study is to determine the effect of allogeneic mesenchymal stem cells on the oncological process in the experimental reproduction of Lewis lung carcinoma.

Literature Review

The principal mechanisms of MSCs influence on the oncological process include stimulation of angiogenesis; changes in the biological properties of tumour cells, specifically sensitivity to apoptosis induction; effect on immunocompetent cells, modulation of epithelial-mesenchymal transition. X. Li *et al*. (2022) noted that carcinoma growth can be considered the result of a "war" or "alliance" due to the intercellular signalling observed between different cell types. Cancer cells secrete chemokines and cytokines, such as CCL2 and TGF-β, which are involved in the activation and retention of mononuclear inflammatory cells and tumour-associated fibroblasts, as well as in the carcinogenic transformation of macrophages. Furthermore, as noted by N. Wu *et al*. (2020), a complex and dynamic interaction occurs after stromal cell retention. MSCs play a key role as they interact with stromal cells and cancer. The multidirectional signals between these cells are mediated by soluble factors, integrins (apoptotic bodies, microvesicles and exosomes), which leads to a special nanocommunication between different types of tumour cells. G. Jothimani *et al*. (2022) proved that exosomes, the smallest subgroup of EVs (30-150 nm in diameter), are surrounded by a protein-phospholipid bilayer membrane, and its lumen partially repeats the contents of the parent cell (DNA, microRNA, matrix RNA (mRNA), growth factors, nucleic acids, chemokines, and cytokines).

G. Jothimani *et al*. (2022) found that under physiological conditions, MSCs produce many exosomes (MSCs-Ex). MSC-Ex include a powerful set of molecules, including more than 150 different microRNAs and more than 850 unique gene products. Thus, MSC-Ex can induce various cellular responses in a wide range of cells that are responsible for modulating many physiological functions. In the study by S. Guo *et al*. (2021) found that MSCs are recipients of impulses from the tumour, but thanks to MSC-Ex, MSCs have the ability to interact with several cell types in the tumour microenvironment, thereby inducing phenotypic and functional changes that can have a significant impact on tumour growth. Similar to MSCs, some types of MSCs-Ex exhibit the ability to support and induce tumour growth, metastasis and invasion, while other MSCs-Ex have antitumour effects, which are related to their origin and tumour type.

Lewis lung carcinoma is a spontaneous tumour isolated from C57BL/6 Margaret Lewis mice and maintained by subcutaneous or intramuscular transplantation since 1951 and is widely used in experimental pathology. In the subcutaneous transplantation of Lewis lung carcinoma culture cells, both the primary tumour and its metastases showed a comparable histological aspect. The primary tumour (primary tumour node) is formed at the transplant site, penetrating the border subcutaneous tissue and muscles. The primary tumour contains large cells without clear differentiation – undifferentiated carcinoma. The cells are large, rounded or irregularly shaped with an eosinophilic cytoplasm and usually a large, rounded nucleus. Atypical mitoses were recorded in each field of view of the slide. In the primary tumour and its metastases, giant cells with one or more hyperchromatic nuclei were detected. Extensive necrosis of the central area of the tumour was recorded. The presence of a thin, unevenly distributed fibrillar stroma can be observed by staining with Gomori reticulin.

H. Kuroda *et al*. (2021) proved that mesenchymal stem cells are localised in breast carcinoma, where they are incorporated into the tumour-associated stroma. However, the involvement of MSCs, including their derivatives, in the pathophysiology of carcinoma has not been considered. The authors found that human bone marrow-derived MSCs, when added to mildly metastatic mammary tumour cells, cause a significant increase in the metastatic activity of cancer cells. If this cellular mixture is injected into the subcutaneous area, the formation of a xenograft tumour is recorded. Breast carcinoma cells increase the de novo supply of the chemokine CCL5 from MSCs, which subsequently has a paracrine effect on cancer cells, stimulating their invasion, motility and metastasis. This enhanced metastatic capacity is reversible and depends on CCL5 signalling through the chemokine receptor CCR5. Taken together, these data provide evidence that the tumour microenvironment promotes the spread of metastases, causing reversible changes in the phenotype of cancer cells.

L. Sun *et al*. (2020) noted that current therapeutic strategies for cancer treatment are usually insufficient to completely destroy malignant cells, as cancer stroma cells are therapeutically resistant. E. Sahai *et al*. (2020) found that cancer-associated fibroblasts (CAFs) primarily represent a heterogeneous type of stromal cell and are important components of the tumour microenvironment (TME). CAFs are the most common type of stromal cells that contribute to tumour progression through specific intracellular mechanisms and their ability to influence other cell types. Recent studies have highlighted a new function of CAF in the remodelling of TMEs, which contributes to tumour progression and affects treatment response through various molecular mechanisms. The complex mechanism of CAF activity, which promotes tumour development, can be used to improve the efficiency of diagnostic and therapeutic procedures.

Materials and Methods

The experiments were conducted on the basis of the scientific laboratory of the Department of Animal Biochemistry and Physiology named after Academician M.F. Gulyi of the NUBiP of Ukraine during 2019-2023 on male mice of the C57Bl/6 line. The mice were 2.0-2.5 months old and weighed 19.0-22.0 g. Mices were kept under standard vivarium conditions with natural light on a full diet, with water freely available. The study followed the ARRIVE guidelines and was conducted according to the guidelines of the EU Directive 2010/63/EU on the protection of animals used for scientific purposes (Directive 2010/63/EU…, 2010; Percie du Sert *et al*., 2020).

For the experimental studies, the tumour process of Lewis lung carcinoma (LCC), which metastasises to the lungs, was modelled. For this, Lewis lung epidermoid carcinoma cells were used, which were obtained from the cell bank of human and animal tissue lines of the R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine (Kyiv).

Before transplantation, Lewis lung carcinoma cells were cultured under standard conditions in Dulbecco's Modified Eagle Medium

(DMEM) with 3-5% foetal bovine serum (FBS) and 1% antibiotic antimycotic (Sigma, USA). Cultivation was carried out in a CO_2 incubator at absolute humidity, 37°C and 5% CO_2. After forming a monolayer of cells, the biomaterial was seeded and cultured under analogous conditions to obtain a sufficient number of cells for transplantation. Cells were suspended using detergents under the control of an inverted Carl Zeiss 40 microscope (Germany).

Obtaining allogeneic mesenchymal cells of red bone marrow culture (BM-AMSCs). These cells were obtained by washing out red bone marrow from the femoral diaphysis of C57/Bl6 mice. Primary material and early passage cells were cultured in disposable plastic culture dishes (Sarstedt, Germany) under standard conditions. The accumulated cellular material was passaged to obtain the required number of cells for transplantation (Kladnytska *et al.*, 2020). The obtained mesenchymal stem cells were analysed by flow cytometry, and the cells were incubated with PE Anti-Mouse CD90.2 (30-H12) antibody (Becton Dickinson, New Jersey, USA) at a dilution of 1:200, according to the manufacturer's instructions (Donnenberg *et al.*, 2013). Data were analysed on a BD FACS Aria cell sorter (Becton Dickinson) using BD FACS Diva 6.1.2 software. A 488 nm laser and 585/42 nm filter were used (Robinson *et al.*, 2023).

The mesenchymal stem cells of the red bone marrow culture of the 3rd passage were tested for viability. The viability of BM-derived aMSCs was determined by staining with trypan blue. A 0.4% aqueous solution of trypan blue (Sigma, USA) was added to the cell suspension, and the mixture was incubated for 2 min. Trypan blue stains dead cells or non-viable cells with damaged membranes blue. The resulting cell suspension was examined in a Goryaev chamber, and under low magnification, live (unstained) cells, as well as dead and damaged (blue) cells were visually counted.

Scheme of the experiment. Lewis lung carcinoma cells were transplanted into male C57/Bl6 mice in the thigh muscle area in the amount of 10^4 cells in 0.5 cm^3 of phosphate-buffered saline. From the moment of cancer initiation, C57/Bl6 mice were divided into three groups. The *first group* included mice with transplanted Lewis lung carcinoma; the *second group* included mice with transplanted Lewis lung carcinoma, which were injected with BM-derived AMSCs in the amount of 1.25×10^4 on Day 8 after tumour cell transplantation. The *third group* included mice with transplanted Lewis lung carcinoma, which were injected with 0.89% NaCl solution (placebo effect).

The course of the tumour process of Lewis lung carcinoma (LLC) was characterised by standard indicators: weight of animals in the experimental groups, latent period of the primary tumour node, parameters of the primary tumour at different periods of tumour growth, the number of mice in the group with lung metastases, the average number of metastases per animal, tumour volume and infiltration of the primary tumour by lymphocytes were determined.

To isolate the mononuclear cell fraction, the method of separating cells of homogenized tumour tissue in a ficoll density gradient was used. The tumour tissue (2 g) from the experimental groups was homogenised. In a centrifuge tube, the tumour tissue homogenate was carefully layered onto ficoll ($\rho = 1.119$ g/mL) and centrifuged at 1,500 rpm. A layer of lymphocytes was collected over the entire cross-sectional area of the tube above the fraction of the ficoll solution, transferred to a clean centrifuge tube, added to Hanks' solution, and precipitated by centrifugation. The supernatant was removed, and the resulting precipitate was resuspended in a certain volume of Hanks' solution. The number of lymphocytes was counted in a Goryaev chamber.

Histological preparations were made to investigate the microscopic structure of muscle tissue at the site of Lewis lung carcinoma primary tumour localisation under the influence of BM-derived AMSCs. For this, fragments of skeletal muscles of the pelvic limb were separated with a sharp blade from the central and peripheral parts of the primary tumour node. Samples were kept for 5 days in a 10% aqueous solution of neutral formalin for fixation. The samples were then rinsed in running water and dehydrated in alcohols of increasing concentration, compacted, and embedded in paraffin on wooden blocks. Sections of skeletal muscle tissue 5-8 μm thick were made on a sled microtome MPS-2. Prepared sections were stained with Carazzi haematoxylin and eosin (Horalskyi *et al.*, 2016). Histological specimens were used to investigate the structure of skeletal muscles at the site of her Lewis lung carcinoma. The area of the tumour parenchyma, vessels, tissue devitalisation areas, and necrotic lesions was calculated by the "point count" method using a Carl Zeiss 40 light-optical binocular microscope (Germany), a Weibel grid for measurements, a MBI-2 microscope and a MOV-1-15^{x} eyepiece micrometer, and UTHSCSA Image Tool software for Windows 7 (version 3.0).

The results were statistically analysed using ANOVA analysis of variance. The results are presented as $x \pm SD$ (mean ± standard deviation). The reliability of the data obtained was assessed by the F-criterion, considering the Bonferroni correction, with a significance level of $P < 0.05$.

Results and Discussion

To conduct the experiment, prior to the study, work was done to obtain mesenchymal stem cells from red bone marrow culture for transplantation. According to the author's findings (Kladnytska *et al.*, 2020), red bone marrow aspirate from the femur and humerus of the limbs of C57/Bl6 mice served as the primary material for obtaining cells with high proliferative activity. The red bone marrow aspirate obtained under sterile conditions was put into centrifuge tubes, phosphate-buffered saline was added and pipetted. Next, the cell suspension was precipitated by centrifugation at 300 g for 20 minutes. The supernatant was removed, and Dulbecco's modified Eagle medium (DMEM) was added to the cell pellet. The cells were suspended and placed in culture dishes for cultivation. The cell suspension was supplemented with 10-15% foetal bovine serum (FBS); 10 μL/cm^3 of antibiotic-antimycotic and cultured in a CO_2 incubator. Cells cultured *in vitro* have discrete properties compared to conventional cells and therefore require a suitable growth medium system. The nature of various environmental factors has a substantial impact on cells in culture. Most often, it is the type of substrate in which cells grow, the components of the culture medium, such as nutrients, chemical and physical properties of the medium, the use of biologically active substances (hormones and growth factors), the temperature of culture incubation, and the manipulations with cells that are provided for by the procedure of obtaining. Subculturing a culture can cause morphological and functional changes when the actual cultured cells change their shape, size, nuclear-cytoplasmic ratio, properties, and the use of such a graft can lead to false results.

The process of cultivation, preservation, and storage of stem cells involves the use of reagents that are not chemically neutral for biological objects. M. Awan *et al*. (2020) noted that dimethyl sulphoxide, which is used as a cryoprotectant to ensure the integrity of cell organoids, is toxic and affects the biological properties of the preserved material. Thus, stem cells obtained under different culture conditions, using additional factors to stimulate proliferation, differ in their biological and functional properties, and in our opinion, they can have different effects in the recipient's body.

Thus, the choice of culture medium, the addition of growth factors for the cultured cells, and the culture conditions are crucial steps in maintaining cell proliferation and vital activity, as well as in preserving the actual morphology and function of the cultured cells. The first adherent cells appeared in the culture dishes after 24-48 hours of cultivation. On Day 3 of cultivation, the formation of mesenchymal stem cell colonies was recorded (Fig. 1).

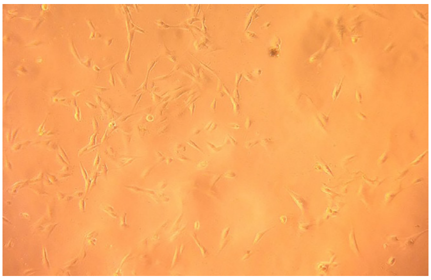

Figure 1. Colonies of mesenchymal stem cells of red bone marrow culture, Day 3 of cultivation

Notes: *native preparation: red bone marrow culture stem cells, × 100*
Sourse*: developed by the author of this study*

As Figure 2 shows, mesenchymal stem cells adhered to the surface of the culture plastic are spread out over the surface and in contact with each other. Under these conditions, contact inhibition of cell proliferation begins, and therefore when the monolayer was 90-100% confluent, a 0.25% solution of trypsin and ethylenediaminetetraacetic acid (EDTA) was added to the culture and left in a thermostat at +37°C for 2 min.

Screening of red bone marrow culture MSC characteristics. Analysis of cytometry data revealed CD90 expression in 69% of cells in the 3rd passage. During the study of the viability of red bone marrow MSCs, it was found that 98% of the cells did not pass the dye through the cytoplasmic membrane and did not stain blue, which confirms the integrity of the cell membrane and their viability. The findings obtained using the flow cytometry method confirmed that the red bone marrow MSC culture met the criteria for MSCs according to the recommendations of the International Society for Cell Therapy (Dominici *et al.*, 2006; Choudhery *et al.*, 2022). V.V. Kovpak & O.S. Kovpak (2018) emphasise that it is important to verify the quality and confirm the characteristics of the obtained cellular material, as this affects its proliferative and functional properties.

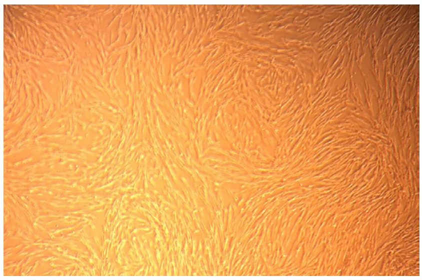

Figure 2. Mesenchymal stem cells of red bone marrow culture, Day 12 of cultivation, monolayer

Notes: *native preparation: red bone marrow culture stem cells, × 100*
Sourse*: developed by the author of this study*

Mesenchymal stem cells are involved in numerous physiological processes, including organogenesis, tissue regeneration or repair, and maintenance of tissue homeostasis. G. Guo *et al.* (2021) recognise that MSCs play an important, dual, and complex role in cancer pathophysiology through their ability to limit or promote tumour progression. Specifically, in gastric cancer, mesenchymal stem cells inhibit the function of normal killer cells through signalling molecules and thus activate tumour progression.

According to C.R. Harrell *et al.* (2021), MSCs interact with the tumour microenvironment, modulate tumour cell behaviour, affect their functions, and promote the formation of distant metastases through the secretion of mediators, modulation of the immune response, and regulation of intercellular interactions. This dynamic system is capable of inducing the formation of immunoprivileged tissue niches or the development of new tumours, which occurs as a result of the differentiation and proliferation of stem cells into tumour-associated fibroblasts and tumour stem cells. At the same time, MSCs have therapeutic effects, including anti-inflammatory, anti-tumour, antioxidant or antiproliferative effects. L. Frisbie *et al*. (2022) reported the ability of MSCs to selectively migrate and penetrate tumour sites. The interaction between tumour cells and non-malignant cells in the tumour microenvironment is a crucial factor in the course of the tumour process. Stromal, immune, endothelial cells, MSCs, which are involved in complex bidirectional cross-interaction with malignant tumour cells, play a crucial role in the directed formation and functioning of the entire microenvironment of tumour tissue. Mesenchymal stem cells are reprogrammed by tumour cells into carcinoma-associated MSCs, which can differentiate into almost all stromal sublines, adipocytes, tumour-associated fibroblasts, and myofibroblasts. Carcinoma-associated mesenchymal stem cells and fibroblasts secrete most of the extracellular matrix in the tumour microenvironment and promote tumour growth and metastasis.

After transplantation of Lewis lung carcinoma cells into C57/Bl6 mice, the time of primary tumour formation in animals of all three groups was almost the same and amounted to 8.2 ± 0.40 days, 8.0 ± 0.35 days, and 8.2 ± 0.40 days, respectively. The body weight of mice with LLC under the influence of BM-AMSCs on Day 14 of the study significantly decreased by 13% ($P < 0.05$) compared to animals of the first and third groups (Table 1).

Table 1. Indicators of tumour diameter and body weight in experimental mice, M ± m, n = 9

Tumour growth period, days	Group 1		Group 2		Group 3	
	body weight of mice, g	tumour diameter, mm	body weight of mice, g	tumour diameter, mm	body weight of mice, g	tumour diameter, mm
8	21.4 ± 0.4	4.8 ± 0.2	20.2 ± 0.7	5.0 ± 0.2	21.3 ± 0.4	4.9 ± 0.3
11	21.3 ± 0.5	5.3 ± 0.5	19.9 ± 0.6	7.3 ± 0.2*^	21.4 ± 0.3	5.2 ± 0.5
14	21.4 ± 0.2	6.2 ± 0.7	18.7 ± 0.6*^	9.5 ± 0.4*^	21.3 ± 0.5	6.3 ± 0.5
18	20.6 ± 0.8	8.1 ± 0.4	18.1 ± 0.4*^	12.3 ± 0.3*^	20.4 ± 0.6	8.2 ± 0.4
24	19.1 ± 0.4	8.9 ± 0.4	17.7 ± 0.3*^	12.8 ± 0.2*^	19.6 ± 0.4	8.5 ± 0.4

Notes: ** – P<0.05, ** – P<0.01, *** – P<0.001 relative to the data in the control group, ^ – P<0.05, ^^ – P<0.01, ^^^ – P<0.001 relative to the data in the placebo group*

Source: *developed by the author of this study*

The body weight of mice in the experimental groups was inversely correlated with the size of the primary tumour node (Table 1). According to the findings, on Day 18 of the study, the tumour diameter increased 1.50 times in mice under the influence of MSCs, and body weight decreased by 12.1% ($P < 0.05$) compared to the control group. Under the influence of MSCs, the diameter of the tumour in mice increased by 1.50 times, while the body weight of the animals decreased by 11.3% ($P < 0.05$) compared to the placebo group.

Subsequently, on Day 24 of the experiment, the dynamics of changes in animal body weight and growth of the primary tumour node was maintained. Significant changes in body weight and diameter of the primary tumour node in mice under the influence of MSCs were recorded. According to the findings of the study, on Day 24 of the experiment, the tumour diameter in mice under the influence of MSCs increased by 1.43 times, and the body weight of the animals decreased by 7.3% ($P < 0.05$) compared to the animals of the control group. The tumour diameter in mice under the influence of MSCs increased by 1.51 times, while the body weight of the animals decreased by 9.7% ($P < 0.05$) compared to the placebo group. Indicators of animal body weight and tumour diameter of group 3 did not significantly differ from those of group 1, which demonstrates the absence of the effect of 0.89% NaCl solution on the promotion of LLC in the classical course.

Z. Jiang *et al*. (2023) noted that tumour-associated macrophages are the main inflammatory component of the stroma of many tumours, which can affect various aspects of neoplastic tissue. It is pericytes located between capillary endothelial cells and the basement membrane that perform the vasomotor function of capillaries, support tissue and organ microcirculation, induce macrophage polarisation, form a pre-metastatic niche, and are an essential component of the tumour microenvironment. S.S. Davidson *et al*. (2020), using RNA sequencing, investigated the diversity of tumour stromal cells at different stages of tumour development. Thus, stromal compartments were identified in murine melanoma and draining lymph nodes (LN) in the locations of tumour formation. Existing lymphocytes from the lymph nodes are activated and cloned within the tumour, while tumour-associated myeloid cells contribute to the formation of a suppressive niche. The authors of the publication emphasised the identification of three temporally distinct populations of stromal cells that demonstrated unique functional characteristics. According to their data, "immune" stromal cells predominate in the early stages of tumour development, and the population of "contractile" cells increases in the later stages of tumour progression. The C3 component of complement is specifically expressed in the immune population. It was found that its degradation product C3a engages C3aR+ macrophages, and changes in C3a and C3aR affect immune infiltration, inhibiting tumour development.

Numerous studies have shown that macrophages in the tumour microenvironment promote angiogenesis, matrix remodelling, and suppression of adaptive immunity, which at the final stage activates the tumour process. H. Kuroda *et al*. (2021) found a positive correlation between an increase in tumour-associated macrophages and a poor prognosis for patients. The researchers point out the significance of the tumour microenvironment. Specifically, an increase in the number of macrophages in the tumour stroma activates intercellular interaction and leads to activation of tumour growth.

Tumour-associated lymphocytes are involved in angiogenesis as producers of vascular endothelial growth factor (VEGF). An increase in the number of tumour-associated lymphocytes confirms the acceleration of the process of vascularisation of the primary tumour and, as a result, metastasis through the circulatory system. The obtained results show analogous data. The number of lymphocytes in the primary tumour tissue under the influence of red bone marrow-derived aMSCs significantly increased by 1.47 and 1.52 times on Day 18, respectively, compared to animals of the control group and placebo, respectively ($P < 0.05$).

Table 2. The number of lymphocytes
in primary tumour tissue, $x10^6$/g tumour tissue, M + m, n = 9

Tumour growth period, days	Group of mice		
	1	2	3
Day 18	1.83±0.35	2.72±0.11*^	1.79±0.29
Day 24	2.13±0.23	2.83±0.35*^	2.11±0.15

Notes: ** – P<0.05, ** – P<0.01, *** – P<0.001 relative to the data in the control group, ^ – P<0.05, ^^ – P<0.01, ^^^ – P<0.001 relative to the data in the placebo group*
Source*: developed by the author of this study*

Comparable results were obtained on Day 24 of the development of the primary tumour node of Lewis lung carcinoma. The number of lymphocytes in the primary tumour tissue under the influence of red bone marrow-derived aMSCs on day 24 significantly increased by 1.33 and 1.34 times compared to control and placebo animals, respectively ($P < 0.05$). This suggests that MSCs create the conditions for vascularisation of the tumour node, and as a result, metastasis by haematogenous means, and, accordingly, activation of the tumour process.

These results are in line with the findings of a study on primary tumour metastasis to the lungs. Notably, in mice of group 2 with LLC, under the influence of red bone marrow-derived aMSCs, the process of lung metastasis was accelerated. This is confirmed by a significantly larger volume of lung metastases, which was 41.52 ± 7.9 mm^3 ($P < 0.05$) compared with the indicators of animals of groups 1 and 3, where they were 17.94 ± 6.59 and 16.43 ± 5.32 mm^3, respectively. Thus, in mice with Lewis lung carcinoma, transplantation of red bone marrow-derived aMSCs significantly affected the process of metastasis and promoted the active transition of lung metastases to the vascular stage.

It was found that MSCs demonstrate high plasticity and undergo functional changes in different microenvironments. Mesenchymal stromal cells are considered to be one of the important components of the tumour microenvironment that induce tumour development. Y. Miyazaki *et al*. (2021) found that MSCs do not inherently promote tumour development, but they acquire tumourigenic properties when exposed to components of the tumour microenvironment. In this context, various elements of the tumour microenvironment that affect the biological characteristics of MSCs through complex interactions are considered: tumour cells, pro-inflammatory factors, immune cells, extracellular matrix, and hypoxia. The researchers noted that in pancreatic ductal adenocarcinoma, cancer-associated fibroblasts are a key component of the proliferating tumour stroma. It was found that adipose tissue culture stem cells can differentiate into separate subtypes of cancer-associated fibroblasts depending on the culture conditions *in vitro*. Therefore, the identification of potential markers of cancer-associated fibroblast subtypes will enable the investigation of the mechanisms of the latter's influence on tumorigenesis.

J. Liu *et al*. (2020) summarised the data on the multifaceted therapeutic functions of MSCs, but in general, they are able to balance the regenerative and inflammatory microenvironment of the affected tissue in the case of severe inflammation. The interaction between MSCs and the immune system was demonstrated by changes in the activity of B lymphocytes. MSCs can induce the formation of regulatory B cells through intercellular contact, soluble factors, and extracellular vesicles. Thus, these cells can complement each other's immunomodulatory functions.

H. Gu *et al*. (2022) noted that extracellular vesicles have a substantial impact on MSCs in the tumour microenvironment, as they carry a variable range of molecules, such as RNA, and can therefore induce reprogramming of MSCs by exosomal oncogenic factors. Exosomes derived from MSCs from various sources, including adipose tissue, bone marrow, umbilical cord, and human olfactory bulbs, showed inhibition of proliferation, migration, and invasion of colorectal cancer cells through regulation of the RAP2B/PI3K/AKT and ITGA2/ITGA6 signalling pathways.

J. Kim *et al*. (2021) investigated methods for obtaining highly purified exosomes from human umbilical cord MSCs and bovine foetal serum based on ultracentrifugation and a tangential flow filtration system. Exosomes derived from human umbilical cord MSCs based on the tangential flow method demonstrated a high angiogenic effect and faster wound healing by 71.4% and 23.1%, respectively.

P. Bule *et al*. (2021) showed that MSCs are exposed to components of the tumour microenvironment and can induce angiogenesis through numerous proangiogenic factors. It was found that after treatment of MSCs with conditioned medium collected from tumour cells, the amount of proangiogenic factors in the tumour microenvironment (mRNA of TGF-β, VEGF, IL-6 and MIP-2) increased. The authors noted that chemokines – chemotactic cytokines – coordinate the exchange between cells in the tumour microenvironment, affect metastasis, angiogenesis, cancer cell proliferation, invasiveness and stemness, and therefore are key dominants in the development of the disease. Many chemokine receptor inhibitors targeting various chemokine signalling pathways are currently being evaluated to modulate the tumour microenvironment and optimise the immune response to overcome patient resistance to chemotherapy. A. Birbrair (2020) reported that MSCs, when interacting with the tumour microenvironment, can directly promote the growth and migration of tumour cells, suppress anti-tumour immunity, and contribute to angiogenesis and overall tumour promotion. Thus, the data presented in this paper on the involvement of MSCs in tumour vascularisation both *in vivo* and *in vitro* coincide with the findings of the above studies.

However, there are well-known scientific studies by H. Gu *et al*. (2021), which proved that MSCs can effectively inhibit tumour progression. The effect of exosomes from MSCs on hepatocellular carcinoma stem cells *in vitro* was investigated. As a result, it was proved that exosomes derived from MSCs block the "malignant behaviour" of hepatocellular carcinoma stem cells due to a considerable reduction in their proliferation, migration, invasion, stimulation of angiogenesis, and self-renewal. This suggests the need to consider the conditions of the experiment. Under these conditions, the MSCs were not exposed to the tumour microenvironment, because the duration of co-cultivation of MSCs with carcinoma cells does not provide the interaction of microenvironmental factors that occurs *in vivo*. *In vivo* hypoxia conditions in tissues also have an impact on tumour development.

S. Bagheri-Mohammadi *et al*. (2020) performed the application of human adipose tissue culture MSCs transfected with TSP-1 factor as a vector therapy for melanoma in mice. As noted above, MSCs affect the tumour process due to their localisation in the microenvironment of tumour tissue. When allogeneic MSCs were injected into bone marrow cultures of animals with Lewis lung carcinoma, the microscopic structure of muscle tissue was noticeably different from that of the control group at all periods of observation.

Under the influence of bone marrow culture MSCs on Day 18 of the study in C57Bl6 mice, the progression of tumour growth in skeletal muscles was found to be greater compared to its spontaneous development. The histological sections revealed that the primary tumour of Lewis lung carcinoma consisted of polymorphic round and oval cells. A nucleus with a large nucleolus occupied almost the entire cell and contained a nucleolus (Fig. 3).

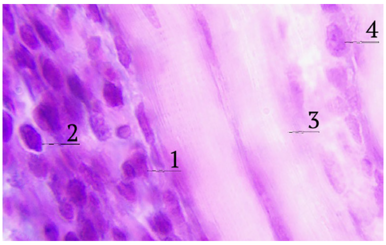

Figure 3. Skeletal muscle of the mouse during the development of primary Lewis lung carcinoma tumour using allogeneic MSCs of red bone marrow culture, Day 18 of the study

Notes: *Histological preparation: 1 – nucleus occupying the entire cell with a large nucleolus; 2 – oval-shaped LLC cells; 3 – longitudinal section of skeletal muscle; 4 – daughter cell of the primary tumour growth. Carazzi hematoxylin and eosin staining, × 400*

Sourse*: developed by the author of this study*

The primary tumour tissue is formed by atypical cells of predominantly round and oval shapes with intensely basophilic large nuclei. The tumour did not have a distinct stroma, which confirms a high degree of its anaplasia. The nuclei of the cells are large, the vast majority of them have a distinct nucleus. The cytoplasm of all tumour cells was intensively stained with Carazzi haematoxylin, which confirms active processes of RNA and, accordingly, protein synthesis. Single giant multinucleated tumour cells were detected. There were signs of metastasis by extension and hematogenous metastasis. These signs are typical for the most malignant tumours and indicate active growth of tumour tissue and progression of the process. On histological sections on Day 18 of the study, under the influence of BM-AMSCs, active metastasis by extension was recorded: the penetration of LLC cells between muscle fibres from the primary tumour node, which formed daughter metastases, was recorded (Fig. 3). There were also relatively large foci of haemorrhage and necrosis of tumour tissue, which were mainly localised in the central part of the tumour, but sometimes also in the peripheral areas (Figs. 4, 5).

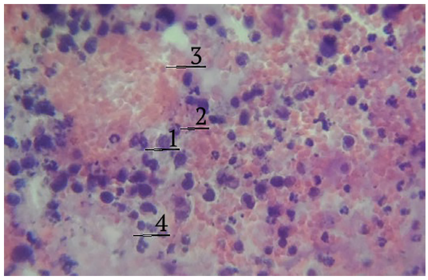

Figure 4. Skeletal muscle of the mouse during the development of primary Lewis lung carcinoma tumour using allogeneic MSCs of red bone marrow culture, Day 18 of the study

Notes: *Histological preparation: 1 – mitosis; 2 – light areas of the nucleus – chromatin; 3 – haemorrhage; 4 – tissue necrosis. Carazzi haematoxylin and eosin staining, × 200*

Sourse*: developed by the author of this study*

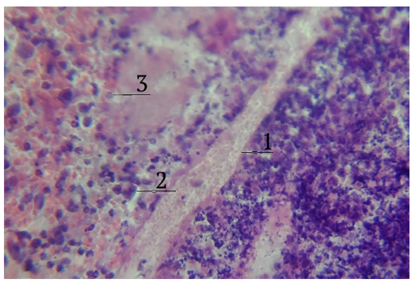

Figure 5. Skeletal muscle of the mouse during the development of primary Lewis lung carcinoma tumour using allogeneic MSCs of red bone marrow culture, Day 18 of the study

Notes: *Histological preparation: 1 – blood vessel; 2 – multinucleated cell; 3 – karyolysis. Carazzi haematoxylin and eosin staining, × 200*

Sourse*: developed by the author of this study*

Notably, on Day 18 of the study, the density of blood vessels in the tumour tissue under the influence of BM-derived aMSCs was 1.4 times higher than in the control and placebo groups. The specific gravity of parenchyma decreased compared to the control group and was 65.5 ± 2.3% ($P < 0.05$). The specific gravity of necrosis areas was almost 2 times higher than that of the control group and amounted to 25.7 ± 4.2 ($P < 0.05$). The studied morphological features of LLC tumour tissue are fully confirmed by the data obtained on the progression of the primary tumour, activation of the lung metastasis under the influence of BM-AMSCs. Studies have shown that MSCs can stimulate the growth of cancer cells and angiogenesis of the primary tumour. In breast and prostate tumours, according to T.E. Krueger *et al*. (2019), MSCs also increased the amount of proangiogenic factors such as VEGF, MIP-2, IL-6, and TGF-β. These factors, as noted by F. Ah-Pine *et al*. (2023), induced tumour cell proliferation and angiogenesis, which substantially accelerated the rate of tumour development *in vitro* and *in vivo*.

Thus, the progression of tumour growth depends entirely on multidirectional signals of soluble factors and intercellular interaction of the tumour microenvironment. Allogeneic MSCs are actively embedded in the tumour stroma, produce exosomes containing many gene products that have an ambiguous effect on a wide range of cells, their phenotype, biological function, induce active cellular responses, and create conditions for nanoconnection of tumour microenvironment components. As a result of this interaction, we observed progression of tumour growth, active vascularisation of tumour tissue, acceleration of metastasis, and its transition to the vascular stage.

Conclusions

The use of allogeneic mesenchymal stem cells from bone marrow culture in C57BL6 mice with Lewis epidermoid metastatic lung carcinoma promoted the activation of the tumour process. After transplantation of Lewis lung carcinoma cells, the period of primary tumour formation in animals of all three groups was practically the same and was 8.2 ± 0.40 days, 8.0 ± 0.35 days, and 8.2 ± 0.40 days, respectively. Under the influence of allogeneic MSCs of red bone marrow culture from Day 14 to Day 24 of the study, the weight of mice decreased by 7.0-12.1% ($P < 0.05$) compared to the control, the diameter of the primary tumour increased by 1.43-1.51 times ($P < 0.05$), which is associated with the activation of primary tumour growth. On Day 18 of the experiment, the number of tumour-associated lymphocytes increased by 1.47 and 1.52 times compared to the control and placebo groups, respectively ($P < 0.05$). Analogous results were obtained on Day 24 of growth of the primary Lewis lung carcinoma tumour. This suggests that MSCs create the conditions for accelerating the vascularisation of the primary tumour and, as a result, metastasis through the circulatory system. Under the influence allogeneic mesenchymal stem cells of bone marrow culture in mice, the process of lung metastasis was accelerated. This is confirmed by a significantly larger volume of lung metastases, which was 1.52 ± 7.9 ($P < 0.05$) compared with the animals of the first and third groups, in which these indicators were 17.94 ± 6.59 mm^3 and 16.43 ± 5.32 mm^3, respectively. The morphological picture of the histological sections of the primary tumour of Lewis lung carcinoma confirms all the signs of qualitative and quantitative indicators of its progression. Under the influence of bone marrow culture MSCs on Day 18 of the study in C57Bl6 mice, the progression of the primary tumour in skeletal muscle tissue was found compared to its spontaneous development. The histological sections revealed that the primary tumour of Lewis lung carcinoma consisted of polymorphic round and oval cells with an intensely basophilic large nucleus and a nucleolus that occupied almost the entire cell. The cytoplasm of all tumour cells was intensively stained with Carazzi haematoxylin, which confirms active processes of RNA and, accordingly, protein synthesis. Single giant multinucleated tumour cells were detected. The tumour did not have a distinct stroma, which confirms a high degree of its anaplasia. There were signs of metastasis by extension and hematogenous metastasis. Relatively large foci of haemorrhage and necrosis of tumour tissue were detected, which were mainly localised in its central part. These signs are typical for the most malignant tumours and indicate active growth of tumour tissue and progression of the process.

In the future, it is planned to determine the influence of allogeneic stem cells from the culture of adipose and nervous tissue on the tumor process.

Acknowledgements

None.

Conflict of Interest

None.

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Вплив алогенних мезенхімних стовбурових клітин культури червоного кісткового мозку на розвиток епідермоідної карциноми легені Льюїс *in vivo*

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Анотація. Актуальність дослідження зумовлена широким використанням стовбурових клітин у ветеринарії та медицині, великим спектром досліджень та неоднозначними даними щодо онкопротекторних властивостей стовбурових клітин різного походження. А тому, мета представленої наукової роботи стосувалася дослідження перебігу пухлинного процесу за карциноми легені Льюїс та особливостей впливу на нього алогенних мезенхімних стовбурових клітин культури червоного кісткового мозку. Провідним підходом дослідження цієї проблеми є метод моделювання карциноми легені Льюїс в мишей C57BL6 та застосування при цьому стовбурових клітин. Використання алогенних мезенхімних стовбурових клітин культури кісткового мозку мишей C57BL6 з трансплантованою епідерміоїдною метастатичною карциномою легені Льюїс сприяло активізації пухлинного процесу. За впливу алогенних мезенхімних стовбурових клітин культури червоного кісткового мозку з 14 до 24 доби дослідження вага мишей зменшувалася на 7,0-12,1 % (P < 0,05) відносно контролю, діаметр первинної пухлини збільшувався в 1,43-1,51 раза (P < 0,05), що обумовлено активізацією росту первинної пухлини. Кількість лімфоцитів, як продуцентів фактору росту судин у первинній пухлинній тканині, за впливу алогенних мезенхімних стовбурових клітин культури червоного кісткового мозку достовірно зростала вже на 18 добу експерименту в 1,47 і 1,52 раза порівняно з тваринами контрольної групи та плацебо (P < 0,05), відповідно. Це сприяло ангіогенезу в первинному пухлинному вузлі та метастазуванню через систему кровообігу. За введення мишам алогенних мезенхімних клітин культури червоного кісткового мозку зареєстровано більший об'єм метастазів у легені, який становив 41,52 ± 7,9 мм^3 порівняно з показниками в тварин контрольної групи та плацебо, відповідно 17,94 ± 6,59 і 16,43 ± 5,32 мм^3. Морфологічна картина гістозрізів первинної пухлини карциноми легені Льюїс підтверджує всі ознаки якісних і кількісних показників її прогресії. Отримані результати становлять як теоретичну, так і практичну цінність для клінічної ветеринарної медицини з питання застосування алогенних мезенхімних стовбурових клітин кісткового мозку за пухлинного процесу

Ключові слова: первинна пухлина; інфільтрація; лімфоцити; метастази; васкуляризація; миші C57BL6