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Influence allogeneic mesenchymal stem cells on the tumour growth parameters and metastatic potential in the transplantable carcinoma lung Lewis

Kladnytska Larysa V[1], Nikulina Viktoriia V[2], Garmanchuk Liudmyla V[2], Mazurkevych Anatoliy Y[1], Kovpak Vitaliy V[1], Nikolaienko Tetiana V[2], Shelest Dmitro V[2], Dzhus Olena I[2], Skachkova Oksana V[3], Stupak Yulia A[2], Dasyukevich Olga I[4]

[1]Ukrainian education And Research Institute of Bioresourses Quality and Life Safety, National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine
[2]Taras Shevchenko National University of Kyiv, Educational and scientific centre "Institute of biology", Kyiv, Ukraine
[3]National Cancer Institute, Kyiv, Ukraine
[4]R. E. Kavetsky Institute of Experimental Pathology, Oncology, and Radiobiology, Kyiv, Ukraine

Email address

KladLarisa@yandex.ru (K. Larysa V), viktorianikulina@ukr.net (N. Viktoriia V), liudmylagarmanchuk@rambler.ru (G. Liudmyla V)

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Abstract

It was investigated the effect of allogeneic mesenchymal stem cell biological characteristics of the primary tumor, metastasis in male mice C57BL/6, which transplanted cell line Lewis lung carcinoma. It was studied the effect of allogeneic MSCs on tumor weight and volume metastasis in tumor-bearing animals, on level of apoptotic cells and tumor cells distribution in cell cycle phases. The primary culture was obtained from transplantable Lewis lung carcinoma. Apoptotic level was measured by flow cytometry, tumor and metastasis volume were assessed by routine methods of experimental oncology, and level of tumor infiltration by lymphocytes was estimated after their fractionation in ficoll-triombrast gradient. We have shown that intravenous administration of allogeneic mesenchymal stem cells after 8 day transplantation of carcinoma lung Lewis leads to enhance of tumor growth in 1.5 times, tumor infiltration by lymphocytes, volume of metastasis in 3 times, and influences on biological characteristics of primary tumor cells, and it leads to an increase in aneuploidy index (in 1,3 times) and mitotic index and reduced levels of apoptotic cells.

Keywords

Allogeneic MSCs, Cell Cycle Phases, Apoptosis, Tumor, Metastasis

1. Introduction

Nowadays stem cells participation in tumor growth has been extensively discussed in anticancer therapy. There are achievements in veterinary and humane medicine. In vitro was investigated, mesenchymal stem cells (MSCs) produced the transient arrest of tumor cells in the G_1 phase of cell cycle; this was accompanied by a reduction in the apoptotic rate [1]. In experiments on rats in vivo was found that the application of xenogeneic MSC, derived from adipose tissue, of experimental models of Guerin's carcinoma significantly increased % survival of experimental animals, and on the 21st day of the experiment at 40% inhibits tumor growth [2]. However, the effects of MSCs on tumor growth are controversial. MSC have the ability to form a cancer stem

cell niche in which tumor cells can preserve the potential to proliferate and sustain the malignant process. Thus, the clinical use of MSCs in conditions in which a malignant disease is involved should be handled with extreme caution [3], [4]. Study on transplantable carcinoma lung Lewis [5] indicates that MSCs promote tumor growth both in vitro and in vivo and suggest that tumor promotion in vivo may be attributable in part to enhanced angiogenesis. But, human MSCs were found to inhibit proliferation of a leukemia cell line and a small cell–lung cancer cell line in vitro, whereas tumor cells grew significantly faster when co-injected with MSCs into nonobese diabetic severe combined immunodeficient mice compared with injection of tumor cells alone [1]. Mesenchymal stem cells are an important component of the tumor microenvironment; however, previous studies have produced controversial results regarding whether MSCs promote or inhibit tumor growth and progression [6], [7]. In view of these controversial data, we conducted a study on the impact of allogeneic MSCs on biological properties of the primary tumor and metastasis rate Lewis lung carcinoma.

2. Main Content

2.1. Material and Methods

The investigation was carried out in C57Bl/6 male mice weighing 20-22g aged 2 to 3 months. All researches on animals were carried out according to Guide for the Care and Use of Laboratory Animals [8].

The strain of metastatic Lewis lung carcinoma (LLC) was kindly given by National Bank of Cell Lines and Tumor Strains of R.E. Kavetsky Institute of experimental pathology, oncology and radiobiology of National Academy of Sciences of Ukraine (IEPOR, NASU). The tumor cell suspension (1 x 10^6 / 0.1ml of Haenk's solution) which was obtained from LLC primary tumor tissues with routine procedure of trypsinization was inoculated intramusculary into mice [9]. After tumor cell inoculation mice were divided into experimental and control groups not less than 8 animals in each. Mice of experimental group received the course of inoculation of MSCs in concentration 1,25x10^4 cells were administrated on 8th day after tumor cell inoculation.

MSCs were obtained from male mice bone marrow. Six-week-old C57BL/6 mice were sacrificed by cervical dislocation. Their femurs were carefully cleaned of soft tissues; epiphyses were removed in sterile conditions; and the bone marrow was harvested by inserting a syringe needle (20-gauge) into one end of the bone and flushing with medium DMEM (Sigma, USA). Cell suspension was layered on ficoll-triombrast with gradient density 1,074 and centrifuged at 300 g 20 min. Then select cell fraction which was on the verge ficoll-triombrast, added Dulbeco's phosphate buffered saline solution, made suspension and centrifugation 5-10 min at 300 g. The cell pellet was resuspended in medium DMEM with the addition 20% FBS (fetal bovine serum) and 1% antibiotic-antimycotic and cultured in standard condition at 37° C, 100% humidity and 5% CO_2. Medium was replaced every 3-4 days. Visual assessment carried out by forming a monolayer every 24 hours using an inverted microscope Axiovert 40 (Carl Zeiss) (Fig.1). When the cells formed a monolayer, it was shooting from the culture dishes 0,25% trypsin containing 0,032% EDTA. The trypsin action was stopped by adding FBS. After centrifugation cells was seeded on the larger diameter cultural dishes and cultivated by the formation of a next passage cells monolayer (Fig.1).

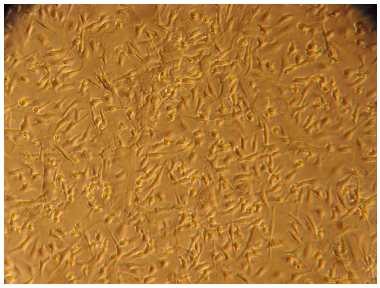

Fig 1. *Mesenchymal stem cells, passage 4, (10x magnification).*

The tumor weight, volume and number of metastases in lung obtained as described [9]. Metastatic volume in lung was assessed by determining the linear dimensions of metastases and their gradation in size ($\leq$0,5mm; 1 mm; $\geq$1,5 mm).

The lymphocyte infiltration degree of tumor (cells/1 g tumor tissue) was estimated after their fractionation on gradient ficoll-verografin with density 1,077 g/ml by centrifugation 40 min at 1.5 thousand rpm.

To determine the effect of MSCs on the biological characteristics of tumor cells on 20th day after tumor transplantation (after 12 days of MSCs administration) primary cultures was obtained. The primary culture was obtained from transplantable Lewis lung carcinoma after 2-3 times trypsinization of tumor tissue in trypsin-EDTA solution with pH 7.0 (Sigma, USA). Cell cultivation was conducted under standard conditions in RPMI medium and 10% FBS (Sigma, USA) at 37° C, 100% humidity and 5% CO_2.

After cultivation under standard conditions in 6-wells plates the number of living cells was determined using MTT-colorymetric test [10] and cell counts were performed using a tripan blue dye after 48 hours incubation tumor cells in primary culture. Apoptotic level and distribution of cells primary culture in phases of cell cycle were assessed by cytofluorimetry [11]. For this purpose the samples were stained with propidium iodide (PI), which selectively joins with intercalating places in DNA. Cytofluorymetry was carried out on the instrument FACS Calibur ("Becton Dickinson", USA). Special mathematical program Mod Fit LT 2.0 (BDIS, USA) for Macintosh computers was used for acquisition and data analysis. Narrow-band filter 585/42 nm was used in order to measure the fluorescence of PI. After cytofluorymetry percentage of aneuploid cells was evaluated in control and after impact of MSCs.

2.2. Results

It is known that the cells of a multicellular organism need to exchange information with each other to regulate its development, the organization of tissues, control of growth and division, to coordinate functions. The bone marrow contains fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, endothelial cells, mesenchymal and hematopoietic stem cells and their descendants. Intercellular interaction occurs via hormones (endocrine cells are formed and reach the target cells through the blood), neurotransmitters (compounds that transmit signals between synapses) and histohormones (out no endocrine cells and have a local effect). Obviously, the latter type of intercellular signal transmission is present between bone marrow cells and the signals received by the bone marrow cells, and report violations of homeostasis in the body arrives via hormonal and neurotransmitter substances. These signals and produce a team that encourages output of a number of stem cells from G_0 phase, which then become the path of differentiation and migrate to the area of the pathological process under the influence of the same intracellular signals. Violation of genetically incorporated a combination of bone marrow cells alters cell-cell interactions and the scheme involves a change in the behavior of cells, which strongly depends on the combination of cells. The results of our previous studies showed that proliferative activity and viability of mesenchymal stem cells is dependent on the method of obtaining the original material and the culture medium [12]. Thus, bone marrow aspirate, which was isolated from the femurs of 6 monthly male mice C57BL/6 was applied to the ficoll gradient density 1,074 and centrifuged at 300 g 20 min to obtain the optimal proportions of bone marrow cells. Such parameters provide a fraction of cells receiving the most enriched MSCs, a combination of bone marrow cells obtained with these parameters is most favorable for high proliferative activity and viability of MSCs.

The resulting fraction of cells derived from bone marrow cultured on medium DMEM. Every day spent assessment of cells adhesion; the formation and growth stem cells colonies. We saw adherent cells on the 4th day of the date of submission cells primary material to medium. And we saw on 12-15th day monolayer formation of MSCs.

Then MSCs were transplanted into spacious the culture dishes to obtain a certain number of MSCs. For intravenous introduction of research groups animal have used 4th passage MSCs.

In determining the parameters of primary tumor growth under MSCs influence we have shown that the rate of growth from 14 days after transplantation (day 8 after transplantation MSCs) tumor volume was increased compared with the control group of animals (Table 1). There was found decrease the body weight of mice after MSCs administration starting from 14th day after tumor transplant and 7 day after the introduction of MSCs compared to the corresponding control.

Table 1. *Animal weight and tumor diameter.*

Day after transplantable tumor LLC cells	Animal weight, g *LLC (control)*	Tumor diameter, mm *LLC (control)*	Animal weight, g *LLC (MSCs)*	Tumor diameter, mm *LLC (MSCs)*
8 th	21.4±0.4		21.2±0.7	
11 th	21.3±0.5	5.3±0.5	20.9±0.6	7.3±0.2
14 th	21.4±0.2	6.2±0.7	19.9±0.6*	9.5±0.4*
17 th	20.6±0.8	8,1 ±0.4	18.9±0.4*	12,3±0.3*
20 th	21.8±0.9		18.3±0.5*	

*p<0.05 *vs* control;

We obtained that the administration of MSCs to the animal with transplantable Lewis carcinoma increased the tumor weight by 41,7% *vs* control, and metastasis volume by 2,8 time *vs* control (Table 2).

Table 2. *Tumor weight and metastasis volume in tumor-bearing animals.*

	LLC (control), n=8	LLC (MSCs), n=8
Tumor weight (g)	2,73±0,29	4,03±0,39*
Metastasis volume (mm^3)	10,3±4,10	29,8±6,10*

*-p<0,05 *vs* control;

This results shows that MSCs increase tumor progression. Especially this effect is expressed by metastasis. Almost about three times enhancement of metastasis may be conditioned by the fact that MSCs enhance the angiogenic potential and create a niche for metastasis, as shown in the works of authors [9], [10].

The size of the metastases differed in both groups. It was found that the number of metastases more than 0.5 mm was increased almost twice after MSCs introduction compared with the control (Fig. 2). Fig.2 is shown example of mice lung with metastases in control and under the influence of MSCs. This results show increase of agiogenesis in metastases under the influence of MSCs. This fact also is confermed by enhancement of tumor infiltration by lymphocytes. Tumor associated lymphocytes are also

Fig 2A. *Lung of animal LLC (control)*

Fig 2B. *Lung of animal LLC (MSCs)*

participants of angiogenesis, as a producers of VEGF. In determining the level of tumor infiltration by lymphocytes significant increase (in 1.3 times) of the indicator in animals with MSCs entered against the relevant controls have been shown (Table 3).

Table 3. *Level tumor infiltration lymphocytes.*

Group of animals	Quantity of lymphocytes, x 10^6/gram of tissue
LLC (control)	1,83 ± 0,35
LLC (MSCs)	2,72 ±0,11*

*-p<0.05 – vs. control;

In the study [14] was shown that the presence of tumor infiltrating lymphocytes has prognostic but not predictive value, and the presence of a dominant nodule in the primary lesion represents a new adverse prognostic factor that should be incorporated in the evaluation of primary melanoma.

In order to determine MSCs influence on biological characteristics of tumor cells they were isolated from primary tumor, incubated 24 hours and then apoptotic level was assessed in control (20 days after tumor transplantation) and test samples (intravenous administration of allogeneic MSCs 4 and 5 passages). As we can see from mentioned data, percent of aneuploidy cells increased under the influence of MSCs (Table 4).

Table 4. *Tumor cells distribution in phases of cell cycle.*

Primary culture	Population quantity, %		% cells in phases of cell cycle			
			G_0/G_1	G_2/M	S	$G_2/M+S$
LLC (control)	*% diploid cells*	40,80+1,70	76,65+4,05	15,81+1,66	6,71+4,87	22,78+3,09
	% aneuploid cells	59,20+1,71	43,82+5,48	10,59+0,08	45,59+5,40	56,09+5,48
LLC (MSCs)	*% diploid cells*	23,30+4,02*	93,18+3,96*	6,65+3,86*	0,16+0,19*	6,81+3,45*
	% aneuploid cells	77,02+3,83*	32,76+7,15	13,54+0,59*	53,72+3,14	67,17+3,65*

*-p<0.05 – vs. control;

The mentioned data revealed that number aneuploid cells increased in 1.3 times upon MSCs influence on transplantable lung Lewis carcinoma. Increase of aneuploidy cells subpopulation in primary culture from animals with the introduction of MSCs indicates the growth of the instability of the genome. According to morphological characteristics, growth of subpopulation of cells of non-adhesion fraction with increase of nucleocytoplasmic ratio was detected in primary culture (Fig. 3A and 3B).

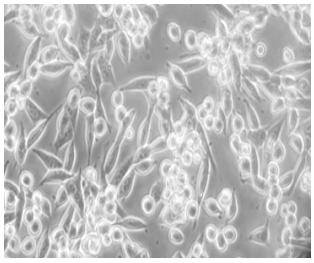

Fig 3A. *LLC cells (control), 20x magnification*

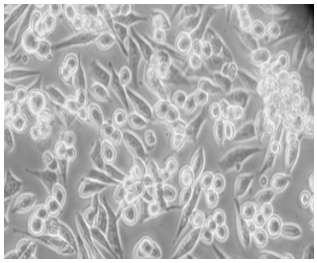

Fig 3B. *LLC cells (MSCs), 20x magnification*

Among aneuploid cells population, increase of cells of proliferative pool were detected after MSCs administration in comparison with control ($G_2/M+S$). As we can see from mentioned data decrease of per cent of diploid cells subpopulation in 1,7 times in primary culture after MSCs administration and their more than 90% synchronization G_0/G_1 phase of cells cycle was shown. Regarding aneuploid cells after MSCs influence, increase of this population to 77,02+3,83% versus 59,20+1,71% in control was accompanied by growth of proliferative pool subpopulation $G_2/M+S$. Thus, biological characteristics of tumor cells under the influence of MSCs are primarily associated with the increase of aneuploidy [15]. Aneuploidy leads to p53 disfunction that may be related to our results about apoptotic level in LLC cells of primary culture under the influence of MSCs (Fig.4).

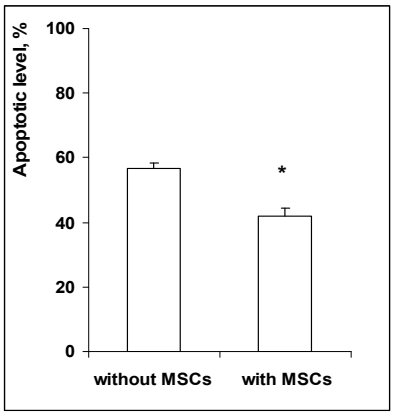

Fig 4. *Level of apoptotic cells.*

The mentioned data revealed that level of apoptotic cells was in 1.4 times decreased in comparison with per cent of apoptotic cells in primary culture without MSCs influence.

3. Conclusion

Thus, intravenous administration of allogeneic mesenchymal stem cells after 8 day transplantation of carcinoma lung Lewis leads to enhance of tumor growth, tumor infiltration by lymphocytes, volume of metastasis and influences on biological characteristics of primary tumor cells, and it leads to an increase in aneuploidy, mitotic index and reduced levels of apoptotic cells. These results suggest

that MSCs play an important role in tumor progression. Thus, the use of stem cells in anticancer therapy needs further investigation.

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