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Antitumor Activity of Rat Mesenchymal Stem Cells during Direct or Indirect Co-Culturing with C6 Glioma Cells

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The tumor-suppressive effect of rat mesenchymal stem cells against low-differentiated rat C6 glioma cells during their direct and indirect co-culturing and during culturing of C6 glioma cells in the medium conditioned by mesenchymal stem cells was studied in an *in vitro* experiment. The most pronounced antitumor activity of mesenchymal stem cells was observed during direct co-culturing with C6 glioma cells. The number of live C6 glioma cells during indirect co-culturing and during culturing in conditioned medium was slightly higher than during direct co-culturing, but significantly differed from the control (C6 glioma cells cultured in medium conditioned by C6 glioma cells). The cytotoxic effect of medium conditioned by mesenchymal stem cells was not related to medium depletion by glioma cells during their growth. The medium conditioned by other "non-stem" cells (rat astrocytes and fibroblasts) produced no tumor-suppressive effect. Rat mesenchymal stem cells, similar to rat C6 glioma cells express connexin 43, the main astroglial gap junction protein. During co-culturing, mesenchymal stem cells and glioma C6 cells formed functionally active gap junctions. Gap junction blockade with connexon inhibitor carbenoxolone attenuated the antitumor effect observed during direct co-culturing of C6 glioma cells and mesenchymal stem cells to the level produced by conditioned medium. Cell–cell signaling mediated by gap junctions can be a mechanism of the tumor-suppressive effect of mesenchymal stem cells against C6 glioma cells. This phenomenon can be used for the development of new methods of cell therapy for high-grade malignant gliomas.

Key Words: *mesenchymal stem cells; glioblastoma multiforme; C6 glioma; conditioned medium*

Modern concept of etiopathogenesis of glioblastoma multiforme, the most malignant glial brain tumor [19,25], is based on the idea that neural stem cells and/or glial progenitor cells can be the source of tumorigenic cancer stem cells that then develop into tumors [12]. Since 2000, the data are accumulated on the potential tumor-suppressive effect of normal neural stem cells and mesenchymal stem cells (MSC) [3,5,8,20,21]. The mechanisms underlying the inhibitory effect of stem cells on the tumor cells are poorly studied. There is evidence that the inhibitory signals from stem cells to tumor cells are transmitted by various secreted factors, *e.g.* bone marrow protein BMP7

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[7] and IL-18 [26]. It is also assumed that gap and adherence junctions between MSC to tumor cells can participate in transmission of the inhibitory signals between the two cell populations [15,18].

The formation of gap junctions (GJ) is described in the majority of mammalian cells. Each contact (connexon) consists of six integral protein subunits, connexins (Cx), arranged in an annular structure forming a pore in the plasmolemma [10]. Connexon can exist as hemichannel maintaining water homeostasis and exchange of low-molecular-weight substances between the cytoplasm and interstitial fluid. Hemichannels of adjacent cells can interact forming a GJ enabling exchange of ions and small molecules (<1000 Da) between the cells [10].

In comparison with other connexin genes, Cx43 gene is actively expressed in high-grade gliomas [6,29]. At the same time, there is evidence that Cx43 is expressed in human MSC [10,20,28]. It is known that heterologous GJ between $Cx43^+$ glioma cells and reactive astrocytes play a role in the active invasion of glioma cells in the peritumoral area [2,15,18]. We have previously demonstrated that both rat MSC and rat C6 glioma cells express Cx43, the main protein of astroglial GJ, and that these two cell populations form functionally active GJ in co-culture [1]. At the same time, the medium conditioned by stem cells can also exhibit antitumor activity [16,30].

Here we compared *in vitro* antitumor activity of rat MSC under conditions of their direct or indirect co-culturing with C6 glioma cells and during culturing of C6 glioma cells in the medium conditioned by MSC to determine the contribution of paracrine factors and direct cell–cell contacts into the antitumor effect of MSC.

MATERIALS AND METHODS

Isolation of MSC from rat bone marrow. Experiments were performed on 4-6-month-old Wistar rats. The bone marrow was isolated as described previously [9] with some modifications. The fraction of mononuclear cells was isolated by centrifugation (1200 rpm, 30 min) in Ficoll density gradient (1.077 g/ml, Sigma-Aldrich). The cells were cultured in DMEM (Gibco) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco), 2 mM GlutaMax (Gibco), and 15% fetal calf serum (FCS; Biowest) in a humid atmosphere at 5% CO_2 and 37°C. Passage 4-5 MSC were used in the experiments. The presence of specific CD markers (CD105, CD90, CD117, CD44 CD45, and CD34) was verified by flow cytofluorometry with appropriate primary labeled antibodies (Mitenyi Biotec).

Rat C6 glioma cells were cultured in DMEM/F-12 (Gibco) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco), 2 mM GlutaMax (Gibco), and 10% FCS (Biowest). Rat astrocytes and Rat2 cells (rat fibroblasts) were cultured in DMEM (Gibco) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco), 2 mM GlutaMax (Gibco), and 5% FCS (Biowest) in a humid atmosphere at 5% CO_2 and 37°C.

Test for pluripotency. Test was performed to confirm that isolated MSC exhibit the properties of stem cells. Cells were cultured in growth media inducing differentiation into adipocytes (StemPro Adipogenesis Differentiation Kit, Gibco), chondroblasts (StemPro Chondrogenesis Differentiation Kit, Gibco), and osteoblasts (StemPro Osteogenesis Differentiation Kit, Gibco). MSC were grown in complete growth medium in 30-mm Petri dishes until confluence and then the growth medium was replaced with differentiation medium. The medium was replaced every 3 days; the test was performed over 21 days.

Preparation of the conditioned medium. Medium was performed as described previously with some modifications. The cells were cultured in DMEM (Gibco) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco), 2 mM GlutaMax (Gibco), and 15% FCS (Biowest) in 25-cm^2 flasks (Corning) in a humid atmosphere at 5% CO_2 and 37°C until 70-80% confluence. Then, the growth medium was removed, the cells were 3-fold washed with Dulbecco's modified PBS, and a fresh portion of growth medium was added. After 24-h incubation in a humid atmosphere at 5% CO_2 at 37°C, the medium was removed from the flask, placed into a 12-ml tube, centrifuged (5 min at 2500 rpm), transferred into a new tube, and used in the experiment. The conditioned medium can be frozen and stored at -80°C. According to this protocol, the media conditioned by MSC (passage 4 minutes), C6 glioma cells (passage 12), rat astrocytes (passage 3), and Rat2 cells (passage 4) were prepared.

Indirect co-culturing of MSC with C6 glioma cells in the Transwell system. Co-culturing was carried out according to manufacturer's protocol (Costar) with some modifications. MSCs were seeded into the upper chambers of a 6-well plate with polycarbonate membrane (10,000 cells/well) in 3 ml growth medium. C6 glioma cells were seeded into lower chambers (5000 cells/well); Rat2 cells not exhibiting signs of stem cells were used as the control (these cells were seeded in the upper chambers (10,000 cells/well). The membrane pore size is 0.4 μ, which excluded cell migration. The experiment was performed in triplicates (3 test and 3 control wells). After 100-h indirect co-culturing, the number of viable C6 glioma cells was analyzed using CellTiter 96 AQueous assay according to manufacturer's protocol (Promega, protocol TB245).

MTS test. The method is based on conversion of MTS-reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to purple formazan crystals catalyzed by mitochondrial dehydrogenases of live cells. Thus, light absorption at λ=490 nm after incubation with MTS reagent is proportional to the number of functionally active cells. MTS test was performed according to the standard protocol [17] with modifications. C6 glioma cell line were seeded in wells of a 96-well culture plate (3000 cells/well). In some wells, the growth medium DMEM (control) was replaced with DMEM conditioned by MSC, Rat2 cells, astrocytes, or rat C6 glioma cells. In some wells, C6 glioma cells were co-cultured with MSC at a ratio of 1:2. GJ blocker carbenoxolone (100 μM) was added to some wells containing co-culture of C6 glioma and MSC. The number of viable cells was evaluated in 24 h using MTS assay (in 4 replicates). The data were processed statistically using Student's *t* test and presented as *M*±*SD*.

Migration test. Migration activity of MSC was evaluated in real time on an xCELLigence RTCA DP cell analyzer (Acea Biosciences) according to manufacturer's guide. The cells were seeded into CIM-Plate wells (an analogue of Transwell system). Experimental wells contained MSC in the upper chambers (20,000 cells/well) and C6 glioma cells in the lower chambers (20,000 cells/well). In controls wells, the lower chamber contained Rat2 cells and rat astrocytes (20,000 cells/well). The instrument measures the resistance on microelectrodes located on the back side of the upper chamber and calculates cell index that is proportional to cell number on electrodes. The migration test was conducted for 20 h. The cells were incubated in a humid atmosphere at 37°C and 5% CO_2. Each experiment was repeated 4 times. Curves describing the time dependence of the cell index were plotted using the averaged results; cell index exceeding 0.5 suggest positive cell migration.

RESULTS

Analysis of stem cells isolated from rat bone marrow revealed the following phenotype: $CD34^-$, $CD44^+$, $CD45^-$, $CD90^+$, $CD105^+$, and $CD117^+$. Expression of CD105, CD90, and CD44 and the absence of CD45 and CD34 suggest that the isolated cell fraction consists of MSC and does no contain admixtures of hemopoietic stem cells (Fig. 1, *a*).

The test for pluripotency showed that MSC cultured in the above differentiation media (StemPro) gradually change their morphological phenotype and acquire features characteristic of adipocytes (lipid inclusions in the cytoplasm), chondroblasts (elongated spindle-shaped cells secreting extracellular matrix), or osteoblasts (cell of irregular shape with processes) (Fig. 1, *b*).

In order to demonstrate the tumor-suppressive effect of the isolated MSC, we evaluated viability of C6 glioma cells after their culturing in MSC-conditioned medium, and after their direct or indirect co-culturing with MSC. The media conditioned by C6 glioma cells, rat astrocytes and rat fibroblasts served as the control in experiments on culturing in MSC-conditioned medium. The medium conditioned by C6 glioma cells was used as the control to exclude depletion of the growth medium as the cause of the cytotoxic effect. To evaluate the contribution of GJ into the antitumor effect of MSC, GJ inhibitor carbenoxolone (100 μM) was added to some wells containing co-culture of C6 glioma cells with MSC. The results of MTS test after 24-h direct co-culturing of MSC and C6 glioma cells showed a significant decrease in the number of live cells (23.2%, $p<0.05$, Fig. 2). Significant differences from the control by the number of viable cells was also found after culturing of C6 glioma cells in MSC-conditioned medium, but in this case, the number of viable glioma cells was higher than after direct co-culturing with MSC (38.5%). In wells where C6 glioma cells were co-cultured with MSC in the presence of carbenoxolone, the number of live glioma cells was 46.9%, *i.e.* higher than without carbenoxolone (Fig. 2).

The number of live C6 glioma cells in wells with medium conditioned by rat astrocytes, fibroblasts, or rat C6 glioma cells did not significantly differ from the control (Fig. 2).

The number of live C6 glioma cells after 100-h indirect co-culturing with MSC decreased to 30.0±3.6% *vs*. 80.60±12.25% in the control ($p<0.05$).

MSC introduced in tumor focus interact with glioma cells [20] and can migrate into the peritumoral area of glioma invasion [4]. Due to their tropism to the tumor focus, stem cells can be considered as a potential cell vector for targeted therapy of gliomas [5,11,22,23]. Cell migration are usually assessed by the rate of monolayer recovery (wound healing assay) or using migration test in the Transwell system (Transwell migration assay) [13,14,24,27]. In the first case, it is very difficult to create a gradient of chemotactic factors modulating migration, and in the second, cell migration cannot be assessed in real time. In our experiments, we assessed migration of MSC towards C6 glioma cells using xCELLigence RTCA DP cell analyzer under conditions of indirect co-culturing that creates a gradient of chemotactic factors; this technique allowed real time counting of migrating MSC with 15-min intervals. It was found that MSC migration towards C6 glioma cells started as early as in 5 h

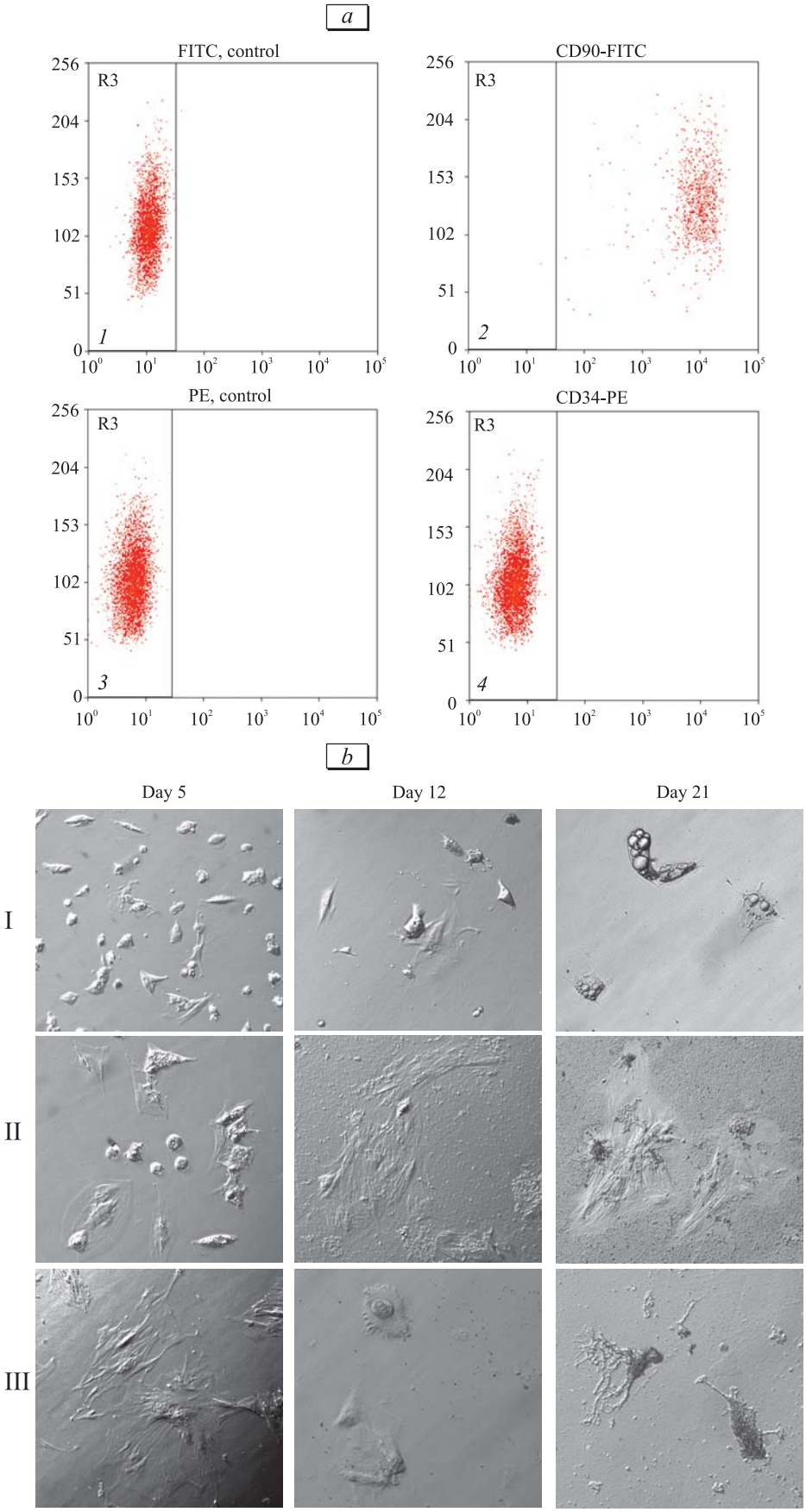

Fig. 1. Immunophenotyping of MSK by flow cytometry (*a*) and test for pluripotency (*b*). *1*) Isotypic FITC-labeled immunoglobulins (negative control); *2*) CD90-FITC (100% cells are positive); *3*) isotypic PE-labeled immunoglobulins (negative control); *4*) CD34-PE (100% cells are negative). Flow cytometry was performed on a MoFlo cell sorter (Beckman Coulter). I: adipogenic differentiation; II: chondrogenic differentiation; III: osteogenic differentiation.

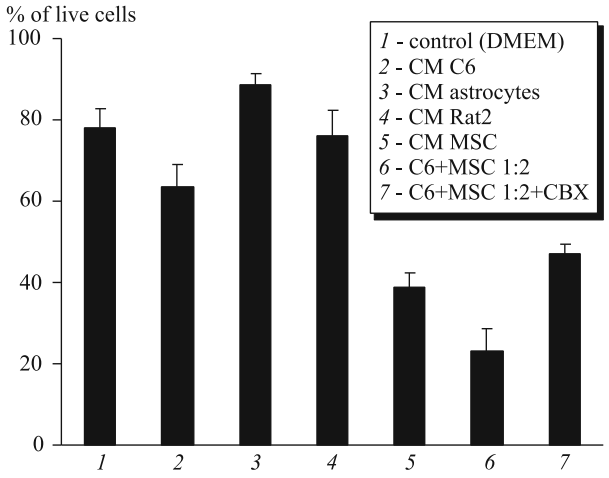

Fig. 2. Quantitative analysis of viability of C6 glioma cells (MTS-test) after 24-h culturing in medium conditioned by C6 glioma cells, rat astrocytes, rat fibroblasts, or MSC, and after direct co-culturing of C6 glioma cells and MSC. CM: conditioned medium, CBX: carbenoxolone.

observed during direct co-culturing of C6 glioma cells and MSCs to the level produced by conditioned medium, which attested to important contribution of direct cell-cell contact between the tumor cells and MSC into realization of the inhibitory effect of MSC. Migration assay showed that MSC actively migrated towards C6 glioma cells, which confirmed the possibility of MSC homing into the tumor after their systemic administration.

Thus, the combination of natural tropism of MSC to glioma cells and their tumor-suppressive activity realized via both paracrine mechanisms and through the formation of GJ with glioma cells make MSC a promising candidate for the development of new methods of targeted therapy of low-differentiated gliomas.

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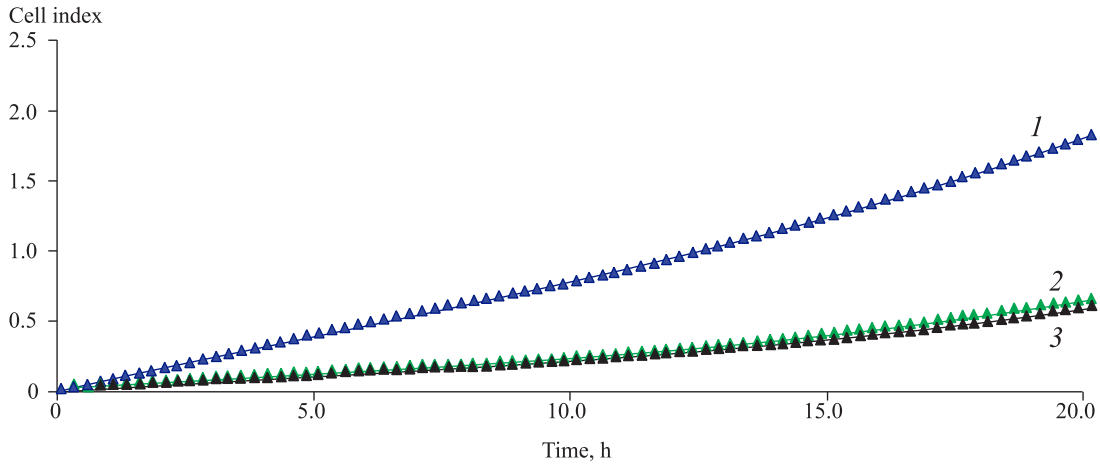

Fig. 3. Analysis of migration activity of MSC towards C6 glioma cells: cell index as a function of incubation time. *1*) Migration of MSC to chambers with C6 glioma cells, *2*) migration of MSC to chambers with rat astrocytes, 3) migration of MSC to chambers with rat fibroblasts.

of indirect co-culturing (Fig. 3) and lasted for 20-25 h. In control wells, positive cell migration was not found throughout the experiment (Fig. 3).

Thus, experiments with direct and indirect co-culturing demonstrated the tumor-suppressive effect of MSC against C6 glioma cells. The most pronounced antitumor activity of MSC was observed during direct co-culturing with C6 glioma cells. When C6 glioma cells were cultured in MSC-conditioned medium, the number of viable glioma cells also significantly decreased in comparison with the control and this cytotoxic effect was not due to exhaustion of the growth medium. The medium conditioned by definitive cell lines (rat astrocytes and fibroblasts) produced no tumor-suppressive effect towards C6 glioma cells. Blockade of GJ with connexon inhibitor carbenoxolone attenuated the antitumor effect

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