In Vitro Inhibitory Effect of Mesenchymal Stem Cells on Zymosan-Induced Production of Reactive Oxygen Species

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In vitro chemiluminescent test showed that human bone marrow mesenchymal stem cells and conditioned media dose-dependently inhibit production of reactive oxygen species by macrophages: 50% inhibition of chemiluminescence (compared to biocontrol) was observed at 1:1 mesenchymal stem cell/macrophage ratio or after addition of 20-25% conditioned media to the incubation medium. The observed mechanism of inhibition of production of reactive oxygen forms can play an essential role in the formation of local immunosuppressive microenvironment in the organism after allogenic transplantation of mesenchymal stem cells.

Key Words: *mesenchymal stem cells; macrophages; reactive oxygen radicals; local immunosuppressive environment*

During recent years, much attention was attracted to the use of cell technologies in medical practice, in particular, to the possibility of local and systemic transplantation of mesenchymal stem cells (MSC) for the replacement of damaged and aged cells and tissues [14,17,20,21]. This can be explained by discovery of MSC plasticity, *i.e.* their capacity to differentiate into cells of various organs and systems of the organism: osteoblasts, chondrocytes, adipocytes, cardiomyocytes, and neuronal cells [14,15]. MSC exhibit unique immunomodulating properties, *e.g.* they can attenuate activity of effector cells of the hemopoietic and immune systems after transplantation into allogenic organism. These properties protect MSC from allogenic rejection in humans and in experimental animals. It is accepted that these mechanisms are related to the following characteristics of MSC:

— hypoimmunogenicity;
— capacity to change T cell phenotype via modulation of dendritic cells (DC) and via direct effect on the function of natural killers (NK-cells) and $CD4^+$ and $CD8^+$ T cells, tissue markers, *etc.*;
— capacity to induce suppressive local microenvironment via production of prostaglandins E2 (PGE2), IL-10, hepatocyte growth factor (HGF), transforming growth factor (TGF-β_1), and by expression of indolamine-2,3-dioxygenase (IDO) [17,20,22].

These characteristics contribute to the creation of local immunosuppressive microenvironment. In the absence of the above listed mechanisms of immunosuppression, transplanted MSC can be subjected to cytotoxic or other influences of effector immune cells of the allogenic organism via induction of the synthesis of perforins, serine proteases,

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IFN-γ, TNF-α, reactive oxygen radicals, *etc*. [1,3, 21]. The mechanisms underlying the suppressive effects of MSC are not quite clear. At the same time, *in vitro* studies with combined culturing of MSC and effector cells allowed to formulate a concept on cell contact-dependent and independent mechanisms of MSC suppression. An important role in these mechanisms is played by modulating effect of MSC on cytokine production by effector cells (macrophages). For instance, inhibition of IFN-γ, IL-12, and TNF-α was demonstrated. These cytokines play a central role in cytokine-mediated immunity [10]. Apart from cytokines, reactive oxygen radicals produced by activated NK-cells, DC, blood monocytes, tissue macrophages, *etc*. also act as active mediators in cell-cell interactions [4,7, 16]. It should be noted that the response in the form of TNF-α production by effector cells develops after 30-40 min, whereas the mechanisms of generation of reactive oxygen species (ROS) are triggered immediately after the action of inductors. At the same time, the problems of possible modification of the level of ROS production by macrophages during combined culturing with MSC or under conditions of *in vivo* transplantation are little studied.

Here we studied *in vitro* modifying effects of human MSC on the level of ROS production by peripheral blood mononuclears from healthy donors (allogenic effects) and mouse peritoneal macrophages (xenogenic effects).

MATERIALS AND METHODS

MSC isolated from the bone marrow of healthy individuals [9] and conditioned media of MSC cultures were used in the study. MSC were cultured in 25-cm^2 Carrel plastic flasks (Sigma); 5×10^6-5×10^7 cells in 8 ml RPMI-1640 growth medium (Sigma) containing 100 U/ml penicillin, 100 ng/ml amphotericin, 2 mM L-glutamine (Sigma), and 20% FCS (Sigma). The medium was changed every 3-4 days. Pooled medium collected during 1-2-week culturing of MSC was used as the conditioned medium (CM).

The modifying effects of MSC cultures or CM on the level of ROS generation by macrophages (phagocytes) during combined culturing were evaluated using *in vitro* test of luminol-dependent chemiluminescence (CL) on a LKB Wallac 1251 luminometer [8].

Mononuclears (lymphocytes and monocytes) and granulocytes (stab and segmented neutrophils) of healthy donors were isolated from 5-7-ml venous blood by centrifugation in Histopaque 1077/1119 density gradient (Sigma) [6,13]. It is accepted that peripheral blood mononuclears act as precursors of tissue macrophages and their activity reflects functional reserved of tissue macrophages [4,5]. Polymorphic nuclears were used as the test control for CL values obtained for mononuclears. It is known that polymorphic nuclear leukocytes as potential blood phagocytes possess powerful mechanisms of ROS hyperproduction for protection of the organism from infections; the level of ROS generation and CL activity of these cells, by one order of magnitude surpass the corresponding parameters of mononuclears and tissue macrophages. These mechanisms of ROS hyperproduction are well studied [1,2,4]. Resident peritoneal macrophages from CBA mice were isolated by peritoneal lavage [6]. Peritoneal macrophages were suspended in cold (4°C) Hanks medium (without phenol red), cell concentration was adjusted to $1\text{-}5\times10^7$/ml, and the cells were stored at melting ice temperature for 3-4 h before use. For evaluation of ROS production, 10^6 isolated human or mouse macrophages in 500 μl Hanks medium containing 10^{-4} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Serva) were placed into luminometer cuvette and 10^6, 10^5, and 10^4 human MSC were added (macrophage/MSC ratio were 1:1; 10:1, and 100:1, respectively). Then, the cell suspension was incubated in cuvette holder of the luminometer at 37°C for 60 min at constant stirring, the intensity of spontaneous (not induced by zymosan) luminol-dependent CL was continuously measured in an automated mode using LKB-Wallac 1254-124 Phagocytosis Program. Opsonized zymosan was added to cell suspension to a final concentration of 1.25 mg/ml. It induced metabolic burst in macrophages and simultaneously enhanced production of extracellular ROS pool [4]. The analysis of zymosan-induced CL of macrophages was continued for the subsequent 40 min (CL peak was measured). Thus, only spontaneous (not induced by zymosan) CL of macrophages modulated by cell-cell interactions was recorded during the first 60 min of co-incubation of MSC and macrophages. Then, zymosan-induced CL was recorded over the subsequent 40 min. The modifying effects of CM from human MSC on CL activity of isolated human blood macrophages and mouse PM were also evaluated. In this case, 10^6 macrophages were added to the incubation medium containing 0, 6, 12, 25, 50, or 100% CM, respectively (and 10^{-4} M luminol). Spontaneous (first 60 min) and zymosan-induced (60-100 min) CL were recorded similarly.

The data are presented as the means of the peak of zymosan-induced CL (in mV/10^6 macrophages) or in relative units (CL index; calculated as the ratio of CL of macrophages under conditions of modifying influence of MSC or CM, respectively, to CL

values measured without modifying influences). Significance of differences was evaluated using Students' *t* test.

RESULTS

Viability and differentiation capacities of transplanted MSC (in the blood stream and during their subsequent contacts with the endothelium, normal cell environment in organ parenchyma, *etc*.) considerably depend on functional activity of effector cells of nonspecific natural immunity (monocytes, neutrophils, macrophages, NK cells, and DC). All these effector cells can respond by metabolic burst associated with the production and release into the environment of ROS producing potent cytotoxic effects: superoxide anion radical, singlet oxygen, H_2O_2, hypochlorite, and NO [1,3-7,16]. Luminol-dependent CL allows quantitative measurements of the concentration of these oxygen radicals in the medium and remains the most highly sensitive and specific tests. It was initially hypothesized that transplanted MSC for maintaining their viability should avoid the influence of the specified effector cells of nonspecific natural immunity or suppress their production. Only in this case, the desirable results of MSC transplantation can be attained.

In our experiments, 10^4-10^6 human MSC added to allogenic mononuclear culture produced weak effect on spontaneous CL activity of macrophages before induction with zymosan, a product obtained from yeast cell walls, inducing a considerable rise of CL activity of macrophages via interaction with C3b and C5a complement fragments and Ig [4] (Fig. 1). The observed minimum changes in CL activity of mononuclears during this period were determined by cell-cell contacts with MSC *in vitro*. On the whole, the observed changes were characterized by the following regularities: the higher was the concentration of added MSC, the lower was the level of spontaneous CL activity of mononuclears. Similar changes in CL activity were detected in xenogenic cell system (human MSC—mouse effector macrophages). Changes in spontaneous CL will not be discussed hereinafter. We will answer two questions: 1) to what extent the CL response of macrophages to an additional stimulus, *e.g.* to opsonized zymosan, is modified by contact with MSC, and 2) whether the response of macrophages depends on the time of their incubation with MSC? Opsonized zymosan induced an almost 10-fold increase in CL of blood mononuclears in the absence of the modifying effect of MSC (Fig. 1). Combined preincubation of MSC and macrophages for 60 min at 37°C considerably suppressed (inhibited)

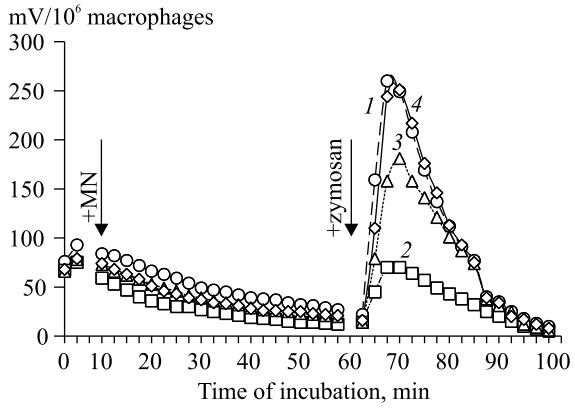

Fig. 1. Effect of concentration of human MSC *in vitro* on CL activity of blood mononuclears (MN) (60-min preincubation before induction of metabolic burst with zymosan). Here and on Fig. 2: mononuclear/MSC ratio in co-culture: *1*) MN, *2*) MN/MSC 1:1, *3*) MN/MSC 10:1, *4*) MN/MSC 100:1.

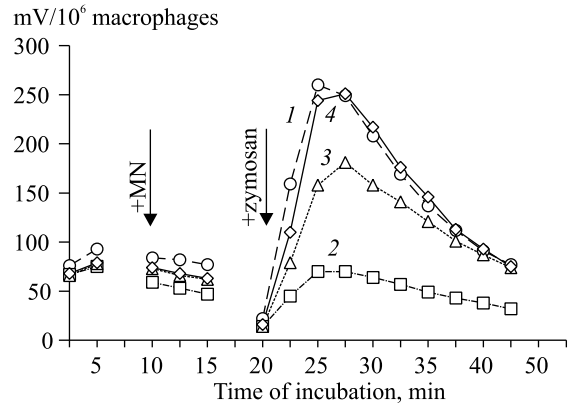

Fig. 2. Effect of concentration of human MSC *in vitro* on CL activity of blood mononuclears (20-min preincubation before induction of metabolic burst with zymosan).

zymosan-induced ROS production by blood mononuclears (Fig. 1). The degree of inhibition appreciably depended on the MSC/mononuclear ratio. The inhibitory effect on ROS production was maximum at 1:1 MSC/mononuclear ratio (differences from biocontrol were significant in this case), and considerably decreased at 1:10 and 1:100 MSC/mononuclear ratio (changes in mean CL activity in these series were insignificant compared to biocontrol). MSC exhibited no spontaneous or zymosan-induced CL activity.

Experimental conditions were changed in further studies: the time of preincubation of MSC with mononuclears was reduced from 60 to 20 min (Fig. 2). It was found that the inhibitory effect of human MSC on zymosan-induced CL activity of blood mononuclears remained practically unchanged. The maximum inhibition of CL-response of mononuclears were also observed at 1:1 MSC/mononuclear ratio, while at 2:1 ratio these effects became more

pronounced and the parameters of CL response were 10-20% of biocontrol. Thus, inhibition of CL response by mononuclears after their interaction with MSC primarily depends on the ratio of these cells *in vitro*, while the time of contact between the effector and stem cells plays no critical role.

It was interesting to find out whether the inhibitory effect of human MSC depends on the nature of effector cells (macrophages). It was shown that increasing concentrations of human MSC *in vitro* similarly decreased CL index of mouse peritoneal macrophages and human blood mononuclears and granulocytes (Fig. 3). In this series, the MSC/mononuclear cell ratio was changed 2-fold (not 10-fold as in the above experiments). The inhibitory effect of MSC on CL-index of effector macrophages little varied and remained within 95% confidence interval (except some points). Hence, irrespective of the initial level of functional activity and some differences in the mechanisms of ROS generation in the studied effector cells [1-4], the degree of CL response most likely depends on the MSC/macrophage concentration ratio in *in vitro* systems and is similar in allogenic (human MSC and blood mononuclears and granulocytes) and xenogenic (human MSC and mouse peritoneal macrophages) mixed cell cultures, *i.e.* does not depend on genetic peculiarities of effectors (macrophages). At the same time, for making definite conclusions we plan to extend our studies on various allogenic, syngeneic, and xenogenic combinations of MSC and macrophages. To this end, MSC cultures isolated from the bone marrow of humans and laboratory animals (rats and mice) will be used.

It can be assumed that MSC can suppress ROS production by macrophages or inactivate ROS in *in vitro* allogenic and xenogenic cell-cell interaction systems. The latter can be a result of immediate cell-cell interactions or mediated influences of mediators released into culture medium on macrophages. Since repeated washout on MSC in Hanks medium always reduced their inhibitory effect on CL-response, we can hypothesize that inhibition of ROS production due to direct cell-cell interactions is mediated via ligand-receptor interactions. Preliminary washout of MSC can reduce the expression of the corresponding ligands on the cytoplasmic membrane and, hence, decrease the inhibitory effect on zymosan-induced CL-response of macrophages. Our experiments showed that CM from human MSC dose-dependently suppress the production of ROS by macrophages (Fig. 4). We analyzed changes in CL-index during incubation of macrophages in the presence of CM *in vitro*: maximum decrease in CL-index was observed in the

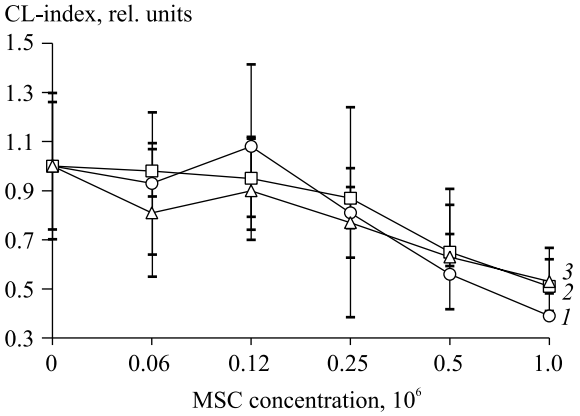

Fig. 3. Effect of concentration of human MSC in vitro on CL-index of mouse peritoneal macrophages (PM) and human blood mononuclears (MN) and granulocytes (GR). *1*) MN+MSC, *2*) GR+MSC, *3*) PM+MSC.

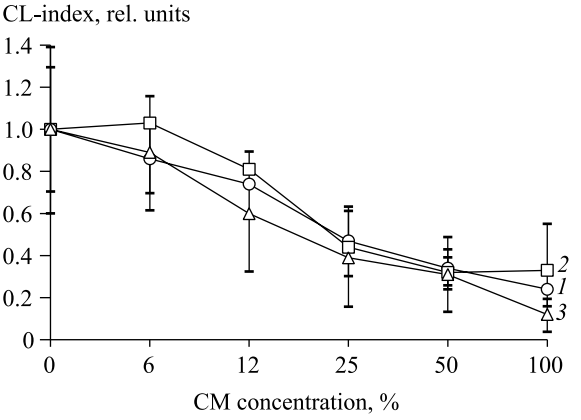

Fig. 4. *In vitro* effect of concentration of CM from cultured human MSC on CL-index of mouse peritoneal macrophages (PM) and human blood mononuclears (MN) and granulocytes (GR). *1*) MN+CM, *2*) GR+CM, *3*) PM+CM.

presence of 100% CM. Decreasing the concentration of CM *in vitro* led to gradual recovery of CL-index to the initial level. Inhibition by 50% (ID_{50}) was observed in the presence of 20-25% CM of human MSC in macrophage incubation medium. The decrease in CL-index little depended on the nature of added macrophages: mouse peritoneal macrophages or human blood mononuclears.

For correct interpretation of the obtained data, we should answer the question, whether or not the decrease in CL-response of macrophages is a result of anergia or apoptosis caused by the cytotoxic effect of MSC or their CM during incubation in mixed culture. To this end, we used galavit, an immunomodulating agent inducing CL-response and 5-7-fold increasing zymosan-induced CL of mono- and polymorphic nuclears of human blood [8]. Thus, after addition of galavit to the incubation medium to a final concentration of 500 μg/ml, the CL index increased by 2.5 times compared to bio-

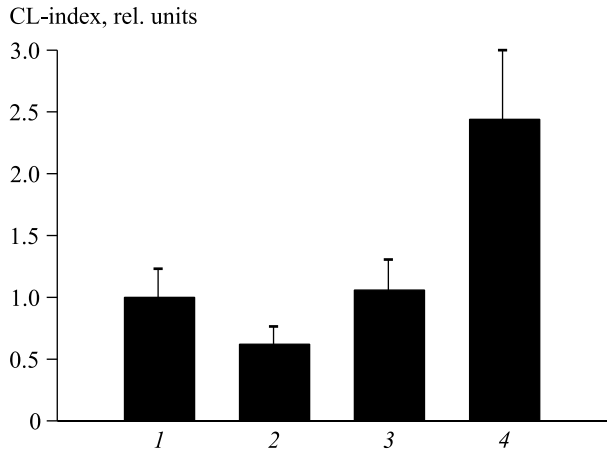

Fig. 5. *In vitro* modifying effect of human MSC and immunomodulator galavit on CL-index of blood granulocytes (GR). *1*) GR without modifying factors (control); *2*) GR /MSC (1:1); *3*) GR /MSC (1:1)+galavit (final concentration 500 μg/ml); *4*) GR+galavit (500 μg/ml) without MSC.

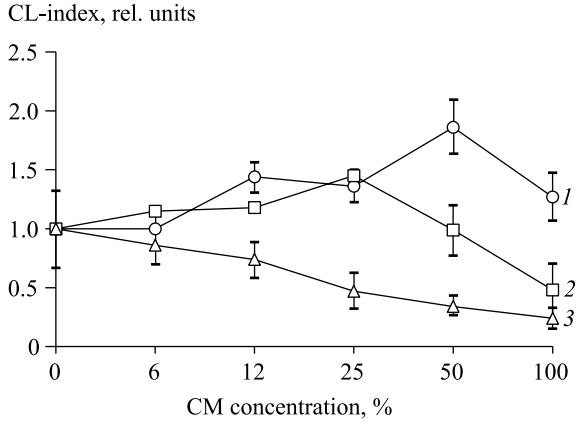

Fig. 6. *In vitro* modifying effect of preliminary 6-fold concentration and dialysis of CM from cultured human MSC on CL-response of mouse peritoneal macrophages (PM). *1*) PM+initial CM (without dialysis); *2*) PM+CM preliminary 6-fold concentrated and dialyzed; *3*) PM+dialyzed CM.

control (Fig. 5). CL-index of granulocytes after incubation with MSC in 1:1 ratio varied from 0.5 to 0.8 rel. units (boundaries of 95% confidence interval). Addition of galavit to mixed culture of MSC and granulocytes restored the CL-response of phagocytes to the level of biocontrol. Similar results were obtained in the MSC/mononuclears+galavit system and in mononuclears (or granulocytes)+ CM+galavit *in vitro* system. These findings attest to viability of phagocytes (granulocytes) in our *in vitro* model and to partially reversible character of the observed inhibition of CL-response of macrophages in mixed culture.

The nature of factor (factors) in CM responsible for inhibition of CL activity of macrophages remains unclear. An attempt was undertaken to determine the molecular weight of this agent. To this end, an aliquot of CM was dialyzed for 12 h at 4°C against 1000x volume of distilled water and then against 10x volume of Hanks saline. Another CM aliquot was 6-fold concentrated by ultrafiltration and then dialyzed as above. Dialysis of CM almost completely abolished the inhibitory effect, while incubation of peritoneal macrophages in the presence of 50% dialyzed CM potentiated the CL-response (Fig. 6). Incubation with 100% non-dialyzed CM reduced CL-index of macrophages to 0.3 rel. units. During incubation of peritoneal macrophages in 100% 6-fold concentrated and dialyzed CM, CL-index was 0.5 rel. units. It is most likely, that the greater part of a low-molecular-weight factor (or factors) is lost during the dialysis. However, some factors can be bound to proteins (*e.g.* PGE2) or they are presented by high-molecular-weigh proteins (cytokines or other protein factors), whose inhibitory effects develop after 6-fold concentration followed by dialysis of CM [22].

We present data on the results of MSC-macrophage interaction during short-term *in vitro* co-culturing. MSC suppress NK-cell proliferation, cytokine secretion, and cytotoxicity [20,21]. Similar inhibition of proliferation and production of some cytokines by MSC is described for mixed lymphocyte cultures [11,18]. Decreased production of IFN-γ, IL-12, and TNF-α in mixed human MSC/monocyte cultures was reported [12]. Similar modification of the cytokine profile was also described for MSC—NK-cell interaction [10]. In this system, the effect modulated by MSC is related to 80% decrease in IFN-γ secretion (at 1:1 cell ratio in co-culture), which is comparable with almost 50% suppression of CL-response of macrophages in our experiments. Previous studies also showed that MSC inhibit IL-15–induced cytokine secretion by NK cells [20]. We showed that at 1:1 MSC/NK-cell ratio in mixed culture, the production of TNF-α and IFN-γ decreased by almost 50%, while secretion of IL-10 by almost 90% compared to the control. Thus, various stages of cytokine secretion by effector cells are modulated as a result of direct cell-cell interactions, which leads to allogenic tolerance and weakening or blockade of T-cell response. The mechanisms of recognition of alloantigens and immunoregulatiry interactions between DC, macrophages, T lymphocyte subpopulations (killers, suppressors, helpers) can be considerably modified in allogenic organism after MSC transplantation. Apart from specific T-cell immunity, an important role in cell recognition independent on the expression of specific alloantigens is played by effector system of nonspecific natural immunity (monocytes, macrophages, NK-cells, neutrophils, DC, and mediators produced by

these cells). Viability and differentiation capacity of transplanted MSC in the allogenic organism, as well as the efficiency of engrafting and growth of tumor cells *in vivo* [5,7] largely depend on activity of effector cells of nonspecific natural immunity.

These findings suggest that MSC grown in bone marrow cultures of healthy patients and their CM produce a dose dependent inhibitory effect *in vitro* on zymosan-induced CL activity of mono- and polymorphic nuclears (granulocytes) from human blood (allogenic combination: human MSC/macrophages) and mouse peritoneal macrophages (xenogenic combination: human MSC/mouse macrophages). The intensity of macrophage CL depends on the level of ROS production [4,7]. Hence, the observed suppression of CL-response resulting from short-term co-culturing of macrophages with MSC is directly related to decreased ROS production by effector macrophages in response to opsonized zymosan inducing metabolic burst. The degree of inhibition of CL-response primarily depends on MSC/macrophage concentration ratio (or concentration of CM from MSC cultures). At MSC/macrophage 1:1 ratio (irrespective of macrophage origin: human blood monocytes or mouse peritoneal macrophages) we observed an almost 50% decrease in zymosan-induced luminol-dependent CL activity of effector cells of nonspecific natural immunity. The inhibitory properties were retained also at MSC/macrophage 1:10 ratio, but were practically absent at a ratio of 1:100. At MSC/macrophage 2:1 ratio, the parameters of CL-response attained only 10-20% of the level of biocontrol. It can be assumed that local concentration of ROS in MSC microenvironment *in vivo* (after transplantation of MSC into allogenic organism) can considerably decrease due to triggering the mechanisms of inhibition of their production. Taking into account the fact that conditioned media produced similar effects and inhibits ROS production, the decrease in the level of ROS in microenvironment of transplanted MSC can be more pronounced than in case of MSC—macrophage contact at 1:1 ratio. Since MSC and CM dose-dependently inhibit ROS production by macrophages, high doses of MSC and CM suppress it, while low doses have no effect or even slightly potentiated zymosan-induced CL-response in mixed MSC-macrophage cultures. It should be noted that macrophages are not at the state of apoptosis or anergy, because they can be *in vitro* restimulated with immunomodulator galavit [8]. Our findings also suggest that CM inhibition can be initiated by low- and high-molecular-weight mediators; the former pass through the dialysis membrane, which leads to partial or complete attenuation of the inhibitory effects, while the latter (high-molecular-weight compounds or low-molecular-weight metabolites bound to proteins) remained in 6-fold concentrated CM after dialysis and determined the inhibitory effects. Moreover, the inhibitory effects of human MSC and CM are not species-specific. It can be assumed that the observed mechanism of inhibition of ROS production is one of the most early stages of modulation of suppressive microenvironment. It can be hypothesized that during evolution multipotent MSC developed different mechanisms of avoidance or minimization of undesirable effects from effector cells of natural immunity, including the mechanisms of cytokine and ROS production. MSC are characterized by their capacities to self-maintenance and differentiation, at least into mature cells (adipocytes, chondrocytes, osteoblasts, *etc*.) [14,18,19]. More differentiated forms of MSC completely retain their immunosuppressive properties of primary stem cells *in vitro* in mixed lymphocyte cultures and partially lose them after transplantation *in vivo* [18]. At the same time, the immunomodulating properties of differentiated MSC (osteogenic or adipogenic cells) *in vitro* considerably depend on the concentration of IFN-γ in the incubation medium [18] or on the number of cells [19]. This suggests that MSC can retain immunomodulating properties, in particular, inhibition of ROS production by tissue macrophages, after differentiation into local cell type in the zone of their grafting (after transplantation). Evaluation of possible mechanisms of these processes *in vitro* on cell cultures and *in vivo* after transplantation of MSC is an actual problem and undoubtedly should be a subject of further studies.

Thus, the following conclusions can be made.

MSC obtained during culturing of human bone marrow cells induce dose-dependent inhibition of zymosan-induced CL in effector cells macrophages. The degree of inhibition of ROS generation primarily depends on MSC/macrophage concentration ratio in mixed culture and practically does not depend on the nature of genetic allogenic or xenogenic combinations of the mixed cells.

CM of human MSC also produce a dose-dependent inhibiting effect on CL activity of effector cells macrophages.

The revealed mechanism of inhibition of ROS production by macrophages (and their active mediators released into the incubation medium) probably plays an important role in the formation of local immunosuppressive microenvironment in the organism after MSC transplantation.

The technique of mixed MSC-macrophage culture with recording of zymosan-induced CL used

by us can be recommended as a method for evaluation of biological activity of MSC in cell therapy of various diseases.

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