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Secretome of Mesenchymal Bone Marrow Stem Cells: Is It Immunosuppressive or Proinflammatory?

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Mesenchymal stem cells (MSC) are characterized by tolerogenic potential and therefore, are used in the treatment of autoimmune diseases such as graft-versus-host disease (GVHD) reactions after allogeneic hematopoietic cell transplantation to improve the transplant functions, as well as for the therapy and prevention of cytokine storm in COVID-19 patients and some other conditions. However, MSC can exhibit proinflammatory activity, which causes risks for their clinical use. We studied the cytokine profile of bone marrow MSC culture and demonstrate intensive production of IL-6, IL-8, and chemokine MCP-1, which participate in the pathogenesis of cytokine storm and GVHD. At the same time, no anti-inflammatory IL-4 and IL-10 were detected. To reduce the risks of MSC application in the GVHD therapeutic protocols, further studies of the conditions promoting generation of MSC with tolerogenic potential and approved clinical standards of MSC use are required.

Key Words: *mesenchymal stem cells; cytokines; graft-versus-host disease; hematopoietic stem cell transplantation*

Multipotent mesenchymal stromal (stem) cells (MSC) are used as a new therapeutic agent for the treatment and prevention of cytokine storm manifestations in COVID-19 and in patients with autoimmune diseases [23]. Another promising application of MSC is prevention and treatment of graft-versus-host disease (GVHD) after transplantation of hematopoietic stem cell and hematopoiesis maintenance under these conditions. However, despite numerous clinical studies of the effectiveness of MSC in various pathologies, no standard recommendations regulating their use were approved, while the results of different trials are ambiguous [10,11,14]. Tolerogenic potential of MSC in the regulation of the immune response are mediated primarily by the spectrum of secreted bioregulators that inhibit activation, proliferation, and effector functions of dendritic cells, macrophages, lymphocytes, and neutrophils [12,13,19]. At the same time, MSC in certain microenvironment can acquire a proinflammatory phenotype, promote activation of neutrophils and T cells, and enhance the immune response [8]. MSC produce anti-inflammatory factors, such as IL-10, prostaglandin E2, and indoleamine dioxygenases (IDO), but can also secrete proinflammatory cytokines such as monocytic chemoattractant protein-1 (MCP-1), IL-6, IL-8, TNFα, and IL-1β. Hence, MSC do not always exhibit immunosuppressive and anti-inflammatory activity. It is assumed that Toll-like receptors expressed by MSC can determine their pro- or anti-inflammatory effects [1,15]. In addition, the duration of MSC culturing is important for their immunomodulatory functions; the researchers suggest that MSC cultures passaged for no more than 3 times are preferable for their clinical use [4].

This study was aimed at evaluation of the spectrum of cytokines secreted during expansion of human MSC for assessing their therapeutic potential.

MATERIALS AND METHODS

Bone marrow aspirates of 8 donors (aged 3-34 years) obtained under sterile conditions were diluted 1:1 with

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α-MEM medium (PanEco), layered onto Ficoll gradient (1.077 g/ml; GE Healthcare), and centrifuged at 450*g* for 30 min to isolate the mononuclear faction. The isolated cells were twice washed with α-MEM at 150*g*, resuspended in complete culture medium on the basis of α-MEM supplemented with 4% platelet lysate, 2 U/ml heparin (Sintez), 0.4 mg/ml alanylglutamine (PanEco), 100 U/ml penicillin, and 100 μg/ml streptomycin (PanEco). The cell suspension was transferred to T25 culture vials (SPL) in a concentration of 10^5/cm^2, and cultured at 37°C and 5% CO_2. After 48-h culturing, non-adherent cells were removed during medium changing. Then, the culture medium was replaced every 3 days. After attaining 80-90% confluence, the cells were harvested with 0.25% trypsin EDTA (PanEco), washed, and transferred to new vials (seeding density 2×10^3 cells/cm^2).

Culture supernatants with secreted cytokines were collected before passage 4 and cytokine contents were measured by ELISA using commercial kits from Vector Best (IL-1β, IL-4, IL-6, IL-8, IL-10, and TNFα) or Abcam (G-CSF, GM-CSF, IL-3, and IL-7). The results were estimated as median values (Me). The supernatants and culture medium samples with 5% platelet lysate were stored at -80°C until analysis.

The expression of MSC surface markers was measured by flow cytometry on an ACCURI C6 cytometer (BD Biosciences) with mouse monoclonal antibodies to human CD90, CD73, CD105, CD34, and CD45 conjugated with fluorochromes FITC and PE-Cy5 (BD Pharmingen). Cell cultures were photographed using the AxioVision 4.0 imaging system (Carl Zeiss).

RESULTS

The primary cultures of adherent cells demonstrate rapid growth and reached 80-90% confluence by day 11-13 after seeding. The cultures had similar morphological characteristics; passage 3 cultures were presented mainly by fibroblast-like spindle-shaped cells and solitary polygonal cells (Fig. 1).

Flow cytometry analysis revealed a highly homogeneous phenotype of the cell culture: >98% cells were positive for MSC antigens: CD90, CD73, and CD105. Markers of hematopoietic cells CD45 and CD34 were expressed by less than 0.9% cells (Fig. 2). Thus, morphological characteristics and the profile of expressed antigens in cultured adherent cells of the bone marrow origin demonstrated high homogeneity and corresponded to the standards for MSC culture [5].

For evaluation of the therapeutic potential of cultured MSC, cytokines important for hematopoiesis maintenance (growth factors G-CSF and GM-CSF, IL-3, and IL-6), as well as cytokines with anti-inflammatory (IL-4 and IL-10) and proinflammatory activities

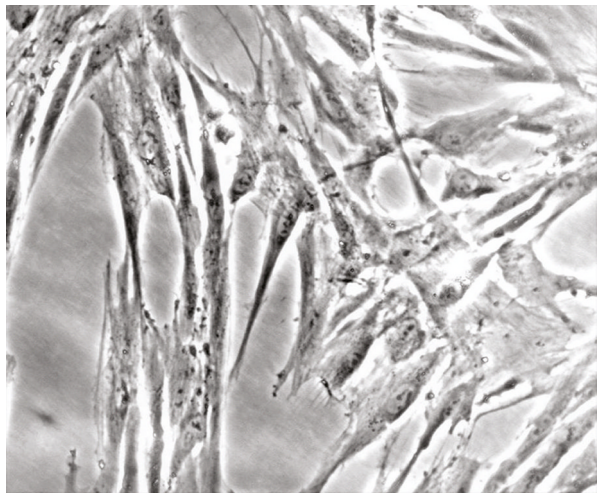

Fig. 1. Culture of bone marrow mesenchymal stem cells, passage 3. Phase contrast, ×400.

(TNFα, IL-1β, IL-6, IL-8, and MCP-1) were analyzed in culture supernatants. None of the samples contained IL-4, IL-10, or TNFα (Table 1). Two samples contained IL-1β (82 and 42 pg/ml) and one sample contained G-CSF (42 pg/ml); IL-3 and GM-CSF were detected in 3 samples, while IL-6, IL-8, MCP-1, and IL-7 were found in all samples, with the highest concentrations of IL-6 and IL-8 (over 5000 and 1000 pg/ml, respectively). However, the concentrations of IL-8 and MCP-1 in the culture medium with 5% platelet lysate was <100 pg/ml and IL-6 was not detected at all.

The obtained data demonstrate individual differences in spontaneous production of IL-3, IL-1β, GM-CSF, and G-CSF and, on the other hand, common spontaneous production of IL-7, MCP-1, IL-6, and IL-8 by MSC from different donors. Anti-inflammatory

TABLE 1. Cytokine Content (pg/ml) in Supernatants of Bone Marrow Mesenchymal Stem Cell Cultures (ELISA Data)

Cytokine	Negative samples (*n*)	Positive samples	
		n	Me (max-min)
IL-6	0	8	15,850 (17,850-5390)
IL-8	0	8	6840 (7180-1130)
MCP-1	0	8	2420 (2560-2270)
IL-7	0	8	20 (37-16)
IL-3	5	3	1480 (1640-1090)
GM-CSF	5	3	19 (34-11)
G-CSF	7	1	42
IL-1β	6	2	42-82
TNFα	8	0	—
IL-4	8	0	—
IL-10	8	0	—

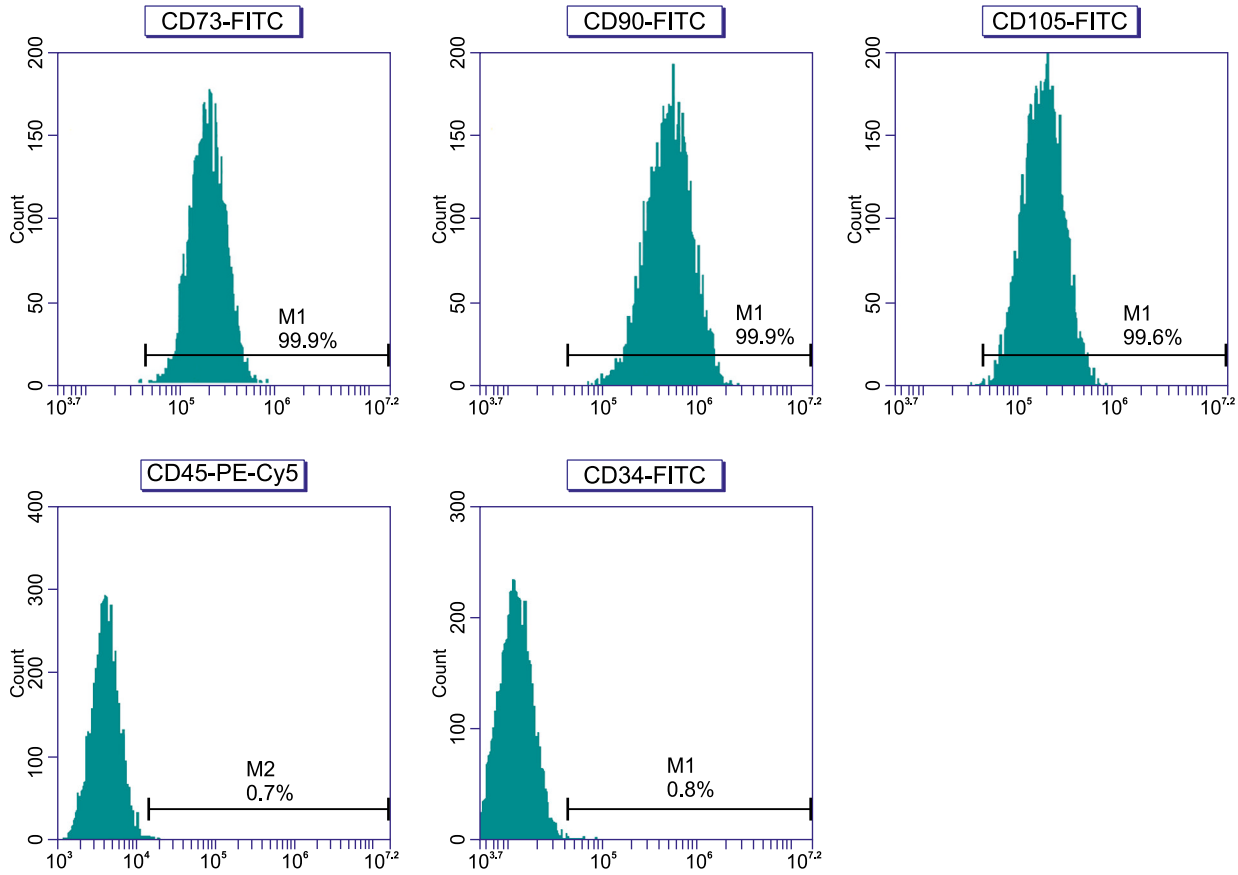

Fig. 2. Expression of the surface antigen in culture of bone marrow mesenchymal stem cells, passage 3.

cytokines IL-4 and IL-10, and proinflammatory TNFα were not detected in the studied cultures.

These results indicate the prevalence of proinflammatory cytokines in the secretome of cultured MSC. In particular, MSC produce significant quantities of IL-6 and IL-8 as well as MCP-1. However, anti-inflammatory cytokines IL-10 and IL-4 were not detected in the supernatants. The results are consistent with previous reports on stable production of IL-6, IL-8, and MCP-1 in the MSC culture until passage 9 [4]. It is important to note that IL-6, IL-8, and MCP-1 are considered the most important factors in boosting the proinflammatory cascade and GVHD [9,22]. Moreover, some studies found that the severity of hyperthermia in GVHD correlates with the levels of IL-6 and IL-8 [7], and current trends in cytokine storm therapy for both GVHD and COVID-19 involve IL-6 receptor inhibitors to block IL-6 signaling [6]. At the same time, the production of IL-6 and other proinflammatory cytokines is an integral feature of MSC, which ensures the undifferentiated status of MSC by inhibiting their adipogenic, osteogenic, and chondrogenic differentiation, maintains their proliferative activity, and prevents apoptosis [18]. The seeming contradiction between the predominance of proinflammatory cytokines in MSC secretome and immunosuppressive activity of MSC can be explained by the fact that IL-6 has the established proinflammatory activity and simultaneously can act as an anti-inflammatory cytokine. Some studies report that IL-6 suppresses the secretion of different proinflammatory cytokines, including IL-1, TNFα, GM-CSF, and IFNγ and induces the production of glucocorticoids, IL-10, an antagonist of the IL-1 receptor, and a soluble receptor for TNFα [17,20]. These data are confirmed by the correlation between IL-6 levels and the immunosuppressive effects of MSC [2]. Along with immunomodulatory activity MSC stimulate hematopoiesis via the production of growth and colony-stimulating factors. Our experiments showed that bone marrow MSC can secrete G-CSF and IL-3. Despite the encouraging results of some clinical trials of MSC-based protocols for GVHD treatment, randomized phase III trials (NCT00562497 and NCT00366145) with commercial MSC (Prochymal) demonstrated their clinical ineffectiveness [16,21]. The National Health Service (England) made a conclusion there is not currently sufficient evidence for the reasonable use of MSC in patients with GVHD [3]. Thus, further studies of the culture conditions for MSC with tolerogenic potential are required for accurate and reliable assessment of

the role of MSC in the therapeutic arsenal of the GVHD treatment and to reduce the risks of MSC clinical use, as well as to develop clinical standards for such therapy.

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