Analysis of Expression of Genes Involved in Immune Response Modulation in Silent Multipotent Mesenchymal Stromal Cells

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The expression of some genes modulating the immune response was studied in multipotent mesenchymal stromal cells (MMSC) from the bone marrow of a healthy donor. Non-activated MMSC expressed IL-6 and IL-10, complement H factor, macrophage growth factor, prostaglandin E2 synthase, and indoleamine-2,3-dioxygenase. The expression of all these genes was higher in female MMSC. A close inverse relationship between IL-6 expression in MMSC and male donor age, close relationship between body weight index and fibroblast CFU concentration in female donor bone marrow and between indoleamine-2,3-dioxygenase and macrophage growth factor in MMSC from these donors were detected. The expression of the analyzed genes was higher in MMSC of donors who had no antibodies to cytomegalovirus, herpes simplex virus, and Epstein-Barr virus in the blood. The results demonstrate the MMSC regulation of immune reactions by MMSC at the cell and organism levels.

Key Words: *multipotent mesenchymal stromal cells; polymerase chain reaction; gene expression; fibroblast colony forming units*

A culture of adherent fibroblast-like cells, so-called multipotent mesenchymal stromal cells (MMSC) and differentiating (under the effect of the relevant inductors) during culturing into various cells of mesenchymal origin (mainly osteocytes, chondrocytes, adipocytes), can be derived from bone marrow cells [2]. The capacity of MMSC to multilineage differentiation, simple procedure of isolation and culturing (in order to increase their quantity and eliminate other than stem cells), and high proliferative potential *ex vivo* suggest the possibility of using these cells for cell therapy and tissue engineering [8].

Attempts at using MMSC and cells obtained as a result of their induced differentiation for the therapy of some diseases have been made. These diseases are imperfect osteogenesis, metabolic disorders, lateral amyotrophic sclerosis, autoimmune diseases [7]. The efficiency of MMSC in the therapy of acute or chronic grant versus host reaction has been demonstrated [9].

It is known that MMSC modulate the immune response *in vitro* and *in vivo* [7]. After stimulation by certain cytokines or direct contact with T cells, antigen-presenting or dendritic cells, MMSC express a wide spectrum of molecules involved in immune response suppression [10], while the data on expression of these molecules in silent MMSC are scanty.

We analyzed the expression of some genes involved in the mechanisms of MMSC interactions with immune cells in silent MMSC (cells which had no contacts with lymphocytes or dendritic cells).

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MATERIALS AND METHODS

The study was carried out on bone marrow specimens from 21 donors (11 women and 10 men, age 13-59 years, mean age 36.0±13.2 years, median 37 years). All bone marrow specimens were obtained by exfusion of donor bone marrow for allogenic transplantation at Department of High-Dose Chemotherapy for Hematological Malignancies and Bone Marrow Transplantation, Hematology Center, after the donors signed informed consent.

Body weight index was calculated by the formula: body weight (kg)/body length (m^2). MMSC were isolated and cultured as described previously [15]. Gene expression was usually analyzed in passage 1 cells.

The incidence of fibroblast CFU (CFU-F) was evaluated for each bone marrow specimen immediately after isolation [14].

Gene expression was evaluated by real-time quantitative Taqman PCR with preliminary reverse transcription.

The RNA was isolated by the standard protocol with slight modifications. The denaturing solution was added directly into culture flasks (pre-washed with phosphate buffer), after which the contents was collected and all the steps were carried out according to the protocol [6]. The first chains of DNA after mRNA hybridization with poly-T-primers were constructed using M-MLV reverse transcriptase (Promega). The target gene was identified by specific primers and

TABLE 1. Nucleotide Sequences, Primer and Test Gene Probe Annihilation Temperatures, and Size of Resultant PCR Products

Gene	Primer, probe	Nucleotide sequence	Annealing temperature, °C
ACTB	Forward primer	CAACCGCGAGAAGATGACC	57.2
	Reverse primer	CAGAGGCGTACAGGGATAGC	56.5
	Probe	ROX-AGACCTTCAACACCCCAGCCATGTACG-RTQ2	72.2
GAPDH	Forward primer	GGTGAAGGTCGGAGTCAACG	58.8
	Reverse primer	TGGGTGGAATCATATTGGAACA	59.3
	Probe	ROX-TGGAATTTCAAATCCAACAAAGTCTGG-RTQ2	67
BMP4	Forward primer	CAC AGC ACT GGT CTT GAG TAT CTT	57.6
	Reverse primer	ACT GGT CCC TGG GAT GTT CTC	59
	Probe	FAM-CA ACA CCG TGA GGA GCT TCC ACC AC-RTQ1	70.4
IL-6	Forward primer	ACCTGAACCTTCCAAAGATG	60.5
	Reverse primer	CTCCAAAAGACCAGTGATGA	59
	Probe	FAM-ATTCAATGAGGAGACTTGCCTGGTG-RTQ1	73
CFH	Forward primer	TTACCCTTACAGGAGGAAATGT	62.5
	Reverse primer	GCTGTCACTGGTAAACACTTC	58.6
	Probe	FAM-CTTCACATATAGGAATATCATTGGTCCAT-RTQ1	70.9
IL-10	Forward primer	CCGTGGAGCAGGTGAAGA	62.8
	Reverse primer	TTGTCATGTAGGCTTCTATGTAGT	60.9
	Probe	FAM-ATAAGCTCCAAGAGAAAGGCATCTAC-RTQ1	69.3
IDO1	Forward primer	AGCGTCTTTCAGTGCTTTG	60
	Reverse primer	GGATTTGACTCTAATGAGCACA	61.4
	Probe	FAM-ACATGCTGCTCAGTTCCTCCAGG-RTQ1	72.3
Ptegs	Forward primer	CTGGTCATCAAGATGTACGTG	59.7
	Reverse primer	CTCCGTGTCTCAGGGCAT	61.6
	Probe	FAM- CTTCTTCCGCAGCCTCACTTGG-RTQ1	73
Csf1	Forward primer	AGGAACTCTCTTTGAGGCTG	60.1
	Reverse primer	CATTCTTGACCTTCTCCAGCA	64.3
	Probe	FAM-CTTGTCATGCTCTTCATAATCCTTGG-RTQ1	71.2

probes (Table 1). All PCR analyses were carried out on Applied Biosystems device.

The gene expression was normalized for each test by the expression of *ACT* (β-actin) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) genes.

The reaction protocol was as follows: preliminary denaturing, 10 min at 95°C; cyclic denaturing, 20 sec at 95°C; hybridization of matrix with primers and elongation, 60 sec at 60°C. Three samples per specimen were analyzed.

Antibodies to CMV, HSV, and EBV were detected using Medac, Euroimmun, and Vector-Best kits.

The data were statistically analyzed by Student's *t* test. The relationships between the analyzed parameters were evaluated by Spearman's nonparametrical coefficient of correlations. The parameters were considered to be in strong correlation at coefficient of correlation >0.7 and in moderate correlation at a coefficient of 0.4-0.7. Only significant ($p<0.05$) values were analyzed.

RESULTS

Donor bone marrow MMSC varied by the pattern of growth in culture and differentiation capacity [5], this indicating their heterogeneity. The group of donors was subdivided by gender. The groups of male and female donors were more or less homogeneous by age, body weight index, and bone marrow concentrations of CFU-F (Table 2). The concentration of CFU-F is often used for evaluating the stromal precursor cells, as the incidence of CFU-F reduces with age, this involving deterioration of MMSC growth parameters [3]. Our data do not confirm this relationship, but we have found that the concentration of CFU-F moderately correlates with body weight index and even strongly so in women (Table 3). Hence, not only hemopoiesis, but also its stromal microenvironment and hence, MMSC depend on the total level of fatty tissue in the body.

The *BMP* (bone morphogenetic proteins) family genes are involved in regulation of the inner media tissue development. The initial level of *BMP4* expression in donor MMSC (an important indicator of MMSC function) was studied [1,5]. This gene was more intensively expressed than genes modulating the immune response and studied in this work (Fig. 1). It was shown on osteoblast strains that the expression of BMP4 was modulated by, for example, adding IL-6 and prostaglandin E2 to culture medium. However, the expression of BMP4 and genes encoding IL-6 and Ptges (enzyme synthesizing prostaglandin E2) genes in the male and female MMSC did not correlate. Presumably, this regulation was realized by the exocrine, but not endocrine way.

TABLE 2. Selective Characteristics for Donors (*M*±*m*)

Parameter	Mean	Men	Women
CFU-F, per 10^6 cells	25.53±5.80	32.52±10.85	19.18±4.80
Age, years	36.00±2.87	37.60±3.75	34.55±4.57
Body weight index	25.69±1.00	27.21±1.42	24.32±1.34

IL-6 is an immunoregulatory cytokine with a wide spectrum of activities: it regulates inflammation and cell division processes, mediates MMSC interactions with monocytes by suppressing their differentiation into dendritic cells. In addition, IL-6 inhibits the dendritic cell maturing and stimulation [10]. The expression of IL-6 in analyzed MMSC from men was more than 2-fold lower, on average, than in MMSC from women, though the differences were negligible (Fig. 1). Moreover, the level of IL-6 expression was closely related and inversely proportional to the age of men, in other words, the level of expression reduced with aging (Table 3); a moderate relationship with age was detected if we compared the data for the total group of donors. No relationship between IL-6 expression and age was detected for female MMSC. It is known that IL-6 stimulates osteoclasts participating in bone tissue remodeling. Its level increases in women during the postmenopausal period (as a rule) characterized by osteoporosis development [11,13]. This can explain the age-specific reduction of IL-6 expression in men, but not in women.

Analysis of the total donor group revealed a moderate relationship between the expression of IL-6 and macrophage growth factor (CSF1), presumably because these factors are essential for monocyte/macrophage differentiation and maybe because their

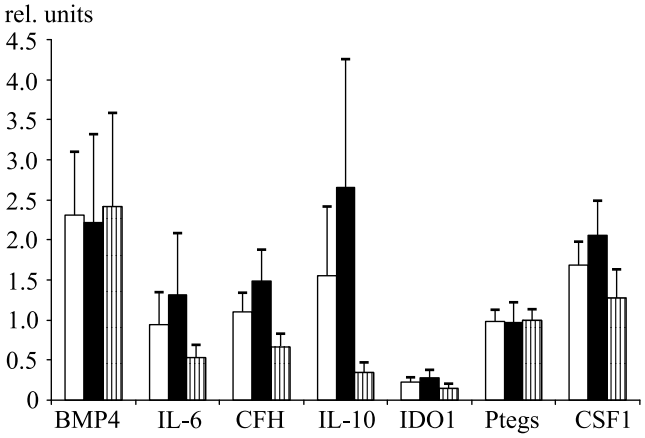

Fig. 1. Level of immune response modulating genes. Light bars: mean; dark bars: in women; cross-hatched bars: in men.

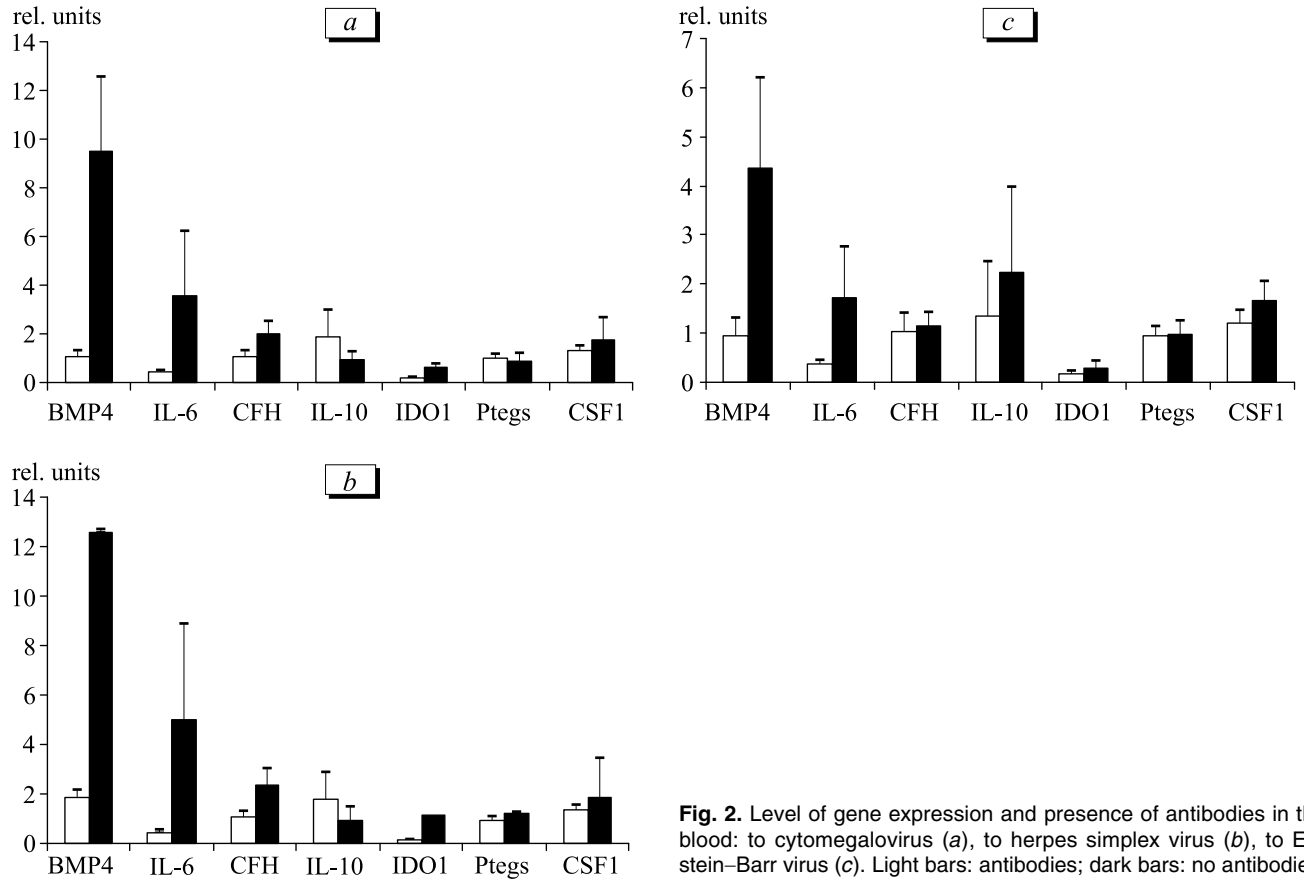

Fig. 2. Level of gene expression and presence of antibodies in the blood: to cytomegalovirus (*a*), to herpes simplex virus (*b*), to Epstein–Barr virus (*c*). Light bars: antibodies; dark bars: no antibodies.

signal pathways are mutually related. It seems that MMSC regulates not only early hemopoietic precursors, but also more mature cells, their differentiation which depending on specific cytokines.

Both male and female MMSC express indoleamine-2,3-dioxygenase (IDO1) at a rather low level. This enzyme inhibits T cell proliferation and is expressed, for example, in some tumor cells, thus causing immune system tolerance of foreign cells. This mechanism is mediated by macrophages and dendritic cells [12]. A strong relationship between IDO1 and CSF1 expression, detected in women, and a moderate relationship between IDO1 and IL-6 expression in the total group of donors demonstrate the functional involvement of MMSC in immune response modulation.

Interleukin-10 is an anti-inflammatory cytokine, its activity synergic with glucocorticoids and other factors modulating the immune response [10]. The expression of IL-10 in the male and female MMSC differs, though insignificantly (Fig. 1). The level of IL-10 expression reduces with increase of prostaglandin E-

TABLE 3. Spearman's Correlations Coefficients for Donor Characteristics, CFU-F Concentrations, and Levels of Gene Expression in MMSC from Bone Marrow of These Donors

Males	Females	Total group
	CFU-F-BWI=0.77	CFU-F-BWI=0.52
IL-6 - Age=-0.78		IL-6 - Age=-0.61
	IL-10 - Ptegs=-0.62	IL-10 - Ptegs=-0.44
		IL-6 - IDO1=0.48
BWI - IDO1=0.64	IDO1 - CSF1=0.78	IL-6 - CSF1=0.44
IL-6 - CFU-F=0.66		CFH - IDO1=0.49

Note. BWI: body weight index. Only significant values ($p<0.05$) are presented.

synthase (Ptegs) expression – a moderate relationship between these parameters has been detected (Table 3).

This enzyme (Ptegs) synthesizing prostaglandin E2 is expressed steadily in the male and female MMSC. It has been shown on the mouse model that endogenous expression of prostaglandin E2 in macrophages disorders the production of IL-6, IL-10, and some other cytokines [4]. It seems that endogenous expression of prostaglandin E2 in MMSC evaluated by expression of the corresponding enzyme (Ptegs) has a similar effect.

The complement factor H (CFH) inhibits stimulation of the complement system during immunological reactions. MMSC express CFH, the level of its expression is 2-fold higher in the female *vs.* male MMSC ($p<0.07$; Fig. 1). In addition, IDO1 and Ptegs inhibit the expression of CFH [16], but we have failed to detect correlations between the expression of CFH and other genes analyzed. In contrast to the products of other studied genes, regulating the immune response at the cell level, CFH is a factor regulating the immune reactions at the organism level. Hence, MMSC, expressing a wide spectrum of regulatory molecules, potentially modulate the immune response directly and at the total systems level through the immune system cells.

The levels of expression of genes involved in immune response were compared in MMSC of donors with antibodies to cytomegalovirus, herpes simplex virus, and Epstein–Barr virus and donors without antiviral antibodies. These viruses were selected because MMSC are more and more often used clinically for the treatment of autoimmune diseases and GVH reaction. These viruses often cause serious complications in patients with weak immune system. The level of *BMP4* expression was reduced significantly in MMSC of all donors who had contacts with these viruses (Fig. 2). Bone marrow concentration of CFU-F in these donors was also slightly reduced (data not presented). It seems that response to contacts with some viruses modulates the physiological status of MMSC. The expression of immunomodulatory cytokines (except IL-10) did not change or reduced in the MMSC of donors who had contacts with these viruses. Reduction of IL-6 expression was the most demonstrative. These data indicate the involvement of MMSC in the host immune reactions and long persistence of changes in the gene expression, detected *ex vivo*.

Hence, silent MMSC express immunomodulatory molecules. Their expression often depends on the gender and immune status of bone marrow donor. The MMSC regulate the immune reactions at the cell and host levels. The findings indicate the significance of personal characteristics of the donor as MMSC source. Allogenic MMSC and their mixtures used for the treatment of diseases should be used responsibly. Further studies of the physiological status of silent and stimulated MMSC are essential for understanding the mechanisms of MMSC activity under conditions of cell therapy.

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